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The calculus of uncertainty

ONE of the most telling headlines to accompany the Three Mile Island nuclear accident appeared over an editorial in the *New York Times* referring simply to "the credibility meltdown". And one of the memories that will linger longest about the accident is the set of uncertainties that surrounded the whole event. There was (and to a large extent remains) uncertainty over the cause of the accident, over the precise danger to people living nearby, over what was happening to the hydrogen bubble that formed above the reactor core, and finally over the long-term effects—whether measured as psychological trauma or as excess cancer incidence—that the accident is likely to have.

Whether, as some have suggested, the sight of experts disagreeing publicly on each of these issues will have any permanent effect on the public perception of scientists is difficult to predict. The image of the infallibility of science is so deeply engrained in our culture that it will take more than one incident to effect any significant change. Indeed, just as the nuclear industry has been turning Three Mile Island to its advantage by claiming, probably justifiably, that the experience will make nuclear power more rather than less safe in the future, so one lesson in the public mind could be the need for more scientific judgement to eliminate the type of uncertainties which seem to have left people so confused.

One of the central dilemmas of advanced societies, however, is that the more technologically sophisticated a culture becomes, the more uncertainties this process generates. We may have visions of evolving into a smoothly run technocracy, with the ability to proclaim, with the American sociologist Daniel Bell, "the end of ideology". But in reality, we are moving into a situation in which, as the debates over how to handle the future development of nuclear power or the relative health risks and benefits of saccharin have clearly demonstrated, the political basis of decision making is becoming more rather than less, important.

We can no longer afford the luxury of believing that greater knowledge, scientific or otherwise, will provide the answers for us. Certainly there is much to be done in developing more sophisticated techniques of technology assessment—of spreading a basic scientific literacy as part of a general education—in analysing situations through various methods of risk/cost/benefit analysis, and so on. But each of these has its limitations, and may only serve

to underline the uncertainties involved in the situations to which they are applied. A report published last week by the US Office of Technology Assessment, for example, which attempted to analyse the potential effects of a nuclear war between the US and the USSR, reached the conclusion that the unpredictable consequences were probably just as great as those which could be predicted, and hence planned for.

There is no simple way out of this situation. Scientists may be partly to blame for creating the pedestals from which others are now trying to shake them—and science educators for encouraging a narrowness of both technical interest and philosophical outlook. But society must take its share of responsibility. A culture which praises individual success rather than collective achievement makes heroes of those, whether film-stars or Nobel prize-winners, who appear to have trodden a lone path to the top, rarely providing equivalent recognition to those who have helped them on their way. While the concentration of decision-making in both the public and the private sector positively discourages substantial questioning by those far from the centres of power.

What this means is that the ability to analyse the complexities raised by advancing technology may be increasingly necessary, but will never be sufficient. Nor will the solution be found in attempts to balance two cultures, the so-called worlds of facts and values respectively. Until we learn to see our apparently technical dilemmas—from the development of an adequate approach to the storage of nuclear waste to acceptable exposure levels for occupational carcinogens—as reflections of political choices, then we shall be incapable of developing solutions that do more than paper over growing cracks.

The judge in the Karen Silkwood case that has just ended in the US made this clear when he directed that a company should be held responsible for the impact of its operations on the health of its employees regardless of whether it had nogens—as reflections of political choices, then we shall which this places on industry is a heavy one and consequently may well be removed by the appeals process. But it is the type of burden that must be acknowledged directly if we are to learn to live with one of the greatest consequences of technological change, the growing of uncertainty to which science can provide guidance, but not answers. □



Scientists debate safety of research on *E coli* strain

A STRONG bid is being made to exempt a large body of recombinant DNA experiments using a disabled strain of the bacterium *Escherichia coli* from the research guidelines laid down by the National Institutes of Health.

Exemption has been proposed to the NIH's Recombinant DNA Advisory Committee (RAC) by Dr Wallace Rowe, head of the Laboratory of Viral Diseases at the National Institute of Allergy and Infectious Diseases, and Dr Allan Campbell, Professor of Biology at Stanford University.

The two scientists base their recommendation on new information which has recently emerged on the potential risks of recombinant DNA research. This evidence, they claim, makes it unreasonable to apply restrictions on most experiments involving the cloning of plasmids inside the 'disabled' *E. coli* K-12 strain, over and above what would normally be considered as safe laboratory practice.

Other members of the committee meeting in Washington last week, however, expressed concern at the implications of such a move. They suggested that, given the many uncertainties still involved, a total exemption from current controls would be premature. However, the committee agreed to set up a working party to study the available data on the safety of experiments using the K-12 strain and suggested that it should consider a proposal that all such experiments are carried out under the minimal P1 physical containment conditions.

In a statement to the committee, Dr Rowe said that information pointing to the safety of K-12 host vector systems included extensive analysis of the biology of the organism and molecular segments cloned inside it, the negative results of monitoring laboratory personnel for acquisition of the bacterium and its plasmids and the results of risk experiments involving the cloning of polyoma virus DNA which he and other research workers had carried out at Fort Detrick last year.

"The basic message from every one of these experiments is that there is no cause for concern about recombinant DNA research with K-12. I do not know of a single piece of new data that has indicated that K-12 recombinant DNA research could generate a biohazard", Dr Rowe said.

For a recombinant organism to become a health hazard, it had to escape from a laboratory, survive outside the laboratory environment, establish itself in some ecological niche, and finally have some detrimental effect on a higher organism. "Since the

original guidelines, data and information have accumulated that indicate that each of these steps is so highly improbable with the K-12 cloning system that each step alone gives sufficient assurance of safety, much less the combination of all four."

Dr Rowe's interpretation of the safety of the K-12 system, however, is not shared by all scientists. In a letter to the RAC commenting on the proposal to exempt most of the experiments using this strain from the guidelines, Dr Roy Curtiss, Professor of Microbiology at the University of Alabama in Birmingham, who has done much work on the development of enfeebled organisms, said he felt such a move would be premature.

In particular, said Dr Curtiss, although it was generally agreed at the Falmouth conference of 1977 that one could not convert K-12 into an epidemic pathogen, a number of studies had since indicated that "the overall probability for transmission of recombinant DNA from *E. coli* K-12 hosts and vectors are higher than I or others believed."

Some of these observations included the excretion of EK1 hosts by human subjects several weeks after receiving a dose, the survival of the disabled λ 1776 strain in the human gut for up to four days, and the selective survival of EK1 hosts in individual mice and rats.

"I surmise that if the participants at the Falmouth conference had been aware of these data, more consideration would have been given to possible consequences of transmission of recombinant DNA to indigenous microorganisms of various natural environments," he said.

In addition to the scientific arguments about the safety of such experiments—other scientists, in particular Dr Jonathan King of MIT and Dr Jon Beckwith of Harvard Medical School have raised the possibility of biologically active recombinant organisms

inducing an auto-immune response from the human body—the committee's discussions also brought in wider considerations.

Dr Rowe has made no secret of his feelings about the guidelines, calling them "wastful, expensive, inefficient, inflexible and inhibitory". The perception or risk need not be zero before restrictions were lifted, he said. "We have to do a cost-benefit analysis. If the guidelines are harming research, which I believe they are, then this has to be entered into the equation". Dr Campbell expressed similar sentiments in support of the proposed exemption. "In my judgement the NIH guidelines as presently written and administered do more harm than good. We should now be examining the guidelines section by section for the hazards that exist."

However, other members of the committee expressed the view that total exemption for a large group of experiments was inadvisable, since risk assessment experiments had not gone far enough to demonstrate that dangers did not exist, while laboratories could not be relied upon to adopt safe procedures (such as a ban on mouth pipetting). "Society is facing a mounting load of hazards, and the RAC is in the vanguard of responsible research in general. You cannot legislate morality, but you can provide a framework for responsibility", said Dr Richard Novick, chairman of plasmid biology at the Public Health Research Institute in New York.

After considerable debate, it was decided to set up a working group to synthesise the data both supporting and not supporting the proposal made by Dr Rowe and Dr Campbell. It was also agreed that the proposal should be modified so that, rather than considering a total exemption for this class of experiments, the committee should consider reducing them to requiring P1 containment levels, as well as registration with local institutional biohazard committees. □

DNA advisers recommend the same controls for industry as universities

RECOMBINANT DNA research carried out by private industry should be legally required to observe the same guidelines as those in force for federally-funded research in university laboratories, the US National Institutes of Health's Recombinant DNA Advisory Committee (RAC) recommended last week.

Members of the committee, which is responsible for advising the director of

NIH on recombinant DNA issues, expressed concern that private companies can register their experiments voluntarily with NIH. (Although many companies are now engaged in such research, only one institution—the Roche Institute of Molecular Biology—has yet asked to be registered.)

"I do not see any justification for maintaining that a system of voluntary compliance that was not deemed

adequate for the scientific community should be adequate for the private sector. Why should you treat the private sector differently?" Dr Sheldon Krinsky, acting director of the programme in Urban, Social and Environmental Policy at Tufts Medical School and a member of the RAC, asked the committee last week.

The committee voted nine to six to support mandatory compliance by non-NIH institutions with the NIH guidelines covering recombinant DNA research. Six members of the committee abstained—NIH Director Dr Donald Frederickson, when appointing individuals to the committee, had made it clear that he expected them to abstain from votes where they might have a conflict of interest (for example, where a scientist was a consultant or a stockholder in a company that might be affected by a RAC decision).

The committee also decided to set up a working party to study containment requirements and related issues for large-scale experiments which would involve both committee members and outside consultants, possibly including trade union representatives. Mr Peter Libassi, General Counsel at the Department of Health, Education and Welfare, met public interest and industry representatives last week to discuss new proposals which the NIH has put forward on how the RAC could guarantee the confidentiality of information which companies would be required to provide in seeking approval for certain types of experiment.

Mr Libassi told both groups that the DHEW will shortly publish in the *Federal Register* the details of the ways in which NIH will register companies on the basis of voluntary compliance with the guidelines. Industry is keen to support such a procedure as an alternative to the regulation of research by the Food and Drug Administration.

However, the public interest groups



"I'm trying to evolve some guidelines that are as weak as we hope our bacteria are!"

at the meeting made it clear that, given the experience of voluntary requirements in other fields such as toxic waste disposal, they felt it unlikely that voluntary compliance would work adequately. Mr Libassi was told that these groups supported legally-backed requirements as recommended by the RAC.

Meanwhile in California, where a number of small companies are now moving rapidly towards large-scale experiments of possible industrial applications of recombinant DNA research, various state legislators, environmentalists and labour representatives have expressed concern over the possible health hazards to workers. The Chairman of the San Mateo Central Labour Council has written to members of the RAC claiming that current federal efforts that rely on voluntary compliance by industry "are no longer adequate to protect the health and safety of recombinant DNA laboratory workers and other Bay Area cities."

Similarly state senator Barry Keene has urged the RAC to deal directly with the "major policy issues posed by increasing industrial development of this new technology". He wants the state to set up a panel of scientists and lay people to study the issue in California.

David Dickson

Northern hemisphere observatory agreement signed in Canary Islands

LAST weekend, Sweden, Denmark and the UK finally signed an inter-governmental agreement with Spain to cooperate over the building of a major observatory at La Palma in the Canary Islands. When it is fully operational (in 1984 at the earliest), the observatory will provide British astronomers with facilities for observing the northern sky equivalent to those at the Anglo-Australian telescope in the southern hemisphere. According to Professor F. Graham Smith, director of the Royal Greenwich Observatory, telescopes at the new observatory could compete in terms of image quality with larger telescopes in the US and Soviet Union, because of the particularly fine seeing conditions at the site.

The UK has been planning to build a 'northern hemisphere observatory' for some years. The Canary Island site was decided on, but progress has been slow because of the long time taken by the Spanish Council of Ministers to put its signature to the intergovernmental agreement. The agreement allows for cooperation in astrophysics and states that Spain will provide the site and facilities in return for some of the telescopes' time and training for Spanish astronomers.

Of the four participating countries, the UK is making the biggest contribution. It is building a new 1m diameter telescope and is planning to move the 2.5m Isaac Newton telescope (INT) from the RGO to the Canaries site. The latter has already been dismantled so that it can be refurbished before shipment next year. The UK Science Research Council, the body involved in the setting up and funding of the observatory, hopes to place a contract for building the domes to house the telescopes this summer.

The UK also has plans for a 4.2m diameter telescope, the largest likely to be built on the site. Although this is only in the design phase and has not yet received the official go-ahead, an order for its mirror blank has already been placed in the US. Even if approval and funds for the whole telescope were given now, however, it would be at least 1984 before it could come into operation. The total cost of the new observatory to the UK, according to Professor Smith, could be about £2.5 million for the 1m telescope, £8 million for moving and refurbishing the INT and in excess of £10 million for the 4.2m telescope.

Sweden's and Denmark's contributions will not be so great. Sweden is planning on transferring, almost immediately, a 16m-high solar tower and a 60cm diameter stellar Cassegrain telescope

Industry spends more on research

Private companies in the US are expecting the total amount of money spent on research and development to be about 13% higher in 1979 than in 1978, according to a survey published last week in New York by McGraw-Hill Publications. Replies from over 500 companies indicated that the total expenditure on R and D would reach \$40,000 million, \$4,600 million more than 1978. Allowing for inflation, this means a real increase of 4.6%.

However the amount of money spent on R and D has declined as a proportion of manufacturing sales, from a peak of 3% in the early 1960s to just over 2%. Over a fifth of research was

directly involved with energy and pollution.

Meanwhile, the National Science Foundation has issued figures showing that the proportion of R and D funding financed by the federal government has fallen below 50% of the total national R and D budget for the first time since full statistics were collected in 1953. The survey suggests this is partly because of cuts in research spending on space and defence and an increased emphasis on civilian R and D programmes. The survey also says that job prospects for scientists and engineers are expected to expand in 1980, particularly in industry. □

to the new observatory. It plans to move its 1.1m Schmidt telescope in two years time. Denmark will collaborate with the RGO over a meridian circle.

In addition to the intergovernmental agreement, two other types of agreement were signed last weekend. One is between the UK Science Research Council, the Swedish Academy of Sciences, the Danish Research Administration and the Spanish Consejo Superior de Investigaciones Científicas. It lays down the details of how the observatory will be used and stipulates the setting up of an international scientific committee to regulate its operation. Each country will be in sole control of 75% of its own telescopes' time. Spain will be entitled to 20% of each telescopes' time and the remaining 5% will be for use by any other country with a stake in the observatory. The UK is also to provide two places each year for Spanish astronomers at British universities, up to four places at the RGO and training at the Canaries observatory. The other agreement, between the Swedish, Danish and British research agencies and the Institute for Astrophysics Research on the Canary Islands, deals with the details of establishing the observatory.

With the opening of the new observatory, most British optical astronomy will move to the Canary Islands. The established centres of optical astronomy in the UK such as the RGO, can adapt to this change, says Professor Smith, by taking leading roles in the management of overseas telescopes, the building and designing of new equipment and in the handling of data from telescopes and spacecraft. In particular, he would like to see the establishment of about a dozen centres for analysing astronomical data with the RGO acting as coordinator. If the SRC were to establish such centres, he says, the UK would be in a good position to act as a data analysis centre for European astronomers using the joint ESA/NASA Space Telescope in the late 1980s.

Judy Redfearn

How the Soviets view exchanges...

"SOVIET-UK scientific contacts are, in general, much worse than 15 years ago, and I am afraid this is mostly the fault of Britain". So Professor Andrei Kapitsa, *de facto* science spokesman of the USSR National Exhibition at Earls Court, London, told *Nature*.

"There are links and agreements", Kapitsa went on, "but somehow they don't work out very well. They can, of course, be improved by mutual efforts, exchanges of publications and mutual experiments, especially in space".

Professor Kapitsa, a geographer working on remote sensing of the environment from space satellites, has perhaps a personal interest in drawing Britain into joint space experiments with the Soviet Union—he was born in England. Moreover, he asserted more than once that the idea of scientific exchange as a one-way flow of information is "absurd"; the idea of the exhibition, he suggested, is to show British scientists and engineers what the USSR has to offer them.

One way of disseminating such information, he explained, was the "Days of Soviet Science and Technology" organised in conjunction with the exhibition. A party of "20 leading Soviet scientists, sponsored by the Royal Society" will lecture in various British universities. This was the first time that such "days" had been organised in Britain, although they had been held in France, Germany, the Comecon countries, he said. (Subsequent inquiries, incidentally, reduced the total number of the party to 11, with the Royal Society being responsible only for three, the remainder, medical personnel, coal and oil technologists, Deputy Minister of Power Sapozhnikov, etc., being cared for by the appropriate professional bodies.)

One purpose of these "days", Kapitsa explained, was to explore the possibility of joint research programmes. His ideas on how exchange should be conducted are firm and somewhat paternalistic.

Exchange of top scientists for short visits would be followed by long-term exchanges of the younger generation "to work under the older ones". When challenged that in the past long-term exchange places were sometimes not filled from the British side, since it was virtually impossible for interested young scientists to find out what individual Soviet institutes had to offer, he merely commented: "But that is what a joint programme is for. The exchange of top scientists provides the necessary information!"

To the suggestion that the flow of such information would be increased if it were made easier for young Soviet scientists to attend international conferences, Kapitsa gave a diplomatic "no". Young people's papers were often not up to standard, and (he implied) could not therefore represent Soviet science. "All they want is an excuse to travel abroad". The American idea that only the younger generation was capable of fruitful scientific ideas was "absolutely stupid", he added. His own father, Nobel laureate Petr Kapitsa, was still doing valuable work in his mid-eighties.

He did admit, however, that the Royal Society and the Soviet Academy, partners in the exchange agreement, did not always operate in the same manner, and made it quite clear which method he thought better. "The Royal Society", he said, "is a society, but the Academy of Sciences is a working board, a research board!"

● Dr R. W. Keay, executive secretary of the Royal Society, comments: "In September 1977, Lord Todd, president of the Royal Society and Academician Anatolii Aleksandrov, president of the Soviet Academy, signed a new exchange agreement, replacing the old rigid system of short-term senior and long-term junior visits by a more flexible arrangement of 49 person-months per year in both directions. Since then the number of exchange visits has virtually doubled." □

... and what they've got to share

KAPITSA'S assertion that the West has much to gain from the maintenance and expansion of exchanges with the Soviet Union reiterates the message of several recent quasi-official Soviet statements. Last month, a lengthy *Pravda* article signed by five members of the Soviet Academy of Sciences (including two vice-presidents and the chief academic secretary) asserted that recent campaigns in the US to sever or suspend scientific contacts with the Soviet Union on human rights grounds are bound to fail and can have no

effect on the progress of Soviet science. An impressive list of Soviet contributions to joint research projects was cited in support.

Last week, Academician Viktor Ambartsumyan, speaking on the North American service of Moscow radio took up the story. While not returning to the Stalinist stance of claiming all discoveries for the Soviet Union, Ambartsumyan claimed that the discovery of the ring of Jupiter by the Voyager mission was not the "complete surprise" claimed in the Western

press; a certain Professor Chashlavskii of Kiev University had predicted it in 1960. The US media, he said, consistently play down the achievements of Soviet science, so as to present exchange programmes as one-sided.

Further, certain US scientists "who probably do not have much political experience", have been "manipulated" into signing a letter making Soviet-US scientific contacts dependent on the fate of "two persons who have been tried and convicted by Soviet courts".

Vera Rich

news in brief

Arabs withdraw bid to ban Israel from WHO: Arab countries last week shelved a resolution which they intended to present to the annual assembly of the World Health Organisation, asking for the suspension of Israel from the agency because of alleged mistreatment of the Arab population in occupied territories. All Arab states except Egypt had pressed for the suspension, which claimed that the Israeli occupation of Arab territories "gravely affects the state of physical, social, psychological and mental health of the population living in these conditions".

However, there were strong pressures from other countries to drop the resolution. In particular, the United States warned that it might withdraw from WHO—to which it contributes about 25% current funds—if the agency's "politicisation" was confirmed by the adoption of the Arab proposal. A panel of experts sent by WHO to the occupied territories last year reported "some improvement" in the medical facilities there, though they could have attained a higher "technical level".

Congress moves to extend moratorium on saccharin ban: A number of bills were introduced into the US Congress last week to extend for a further three years a moratorium on the banning of the artificial sweetener saccharin which was imposed by Congress eighteen months ago. The purpose was partly to allow more time to collect scientific data on the effects of saccharin, which had been shown to cause cancer in laboratory animals, but which is also claimed to have health benefits.

The moratorium expired last Wednesday. In the light of a recent report by the National Academy of Sciences, showing that saccharin posed a special health risk to children because of their high consumption of soft drinks, Dr Donald Kennedy, Commissioner of the Food and Drug Administration, said last week that he was opposed to extending the moratorium. "Chronic consumption, begun early in life, may significantly increase one's risk of developing cancer as an adult," he said, adding that, rather than a further moratorium, he would prefer legislation outlawing the use of saccharin in diet soda, but allowing its continued use in certain types of food.

HSE reports on Windscale leak: The UK Health and Safety Executive has issued an interim report on the status of the Windscale leak to the Secretary of State for Energy. The HSE has found that additional radioactive material has reached the west side of building B212. The new contamination is "much smaller and localised than on the East side of the building" the report says but the soil above the contaminated region is providing shielding against gamma radiation and no excess radiation has been recorded on the surface. The HSE regards the leak as being "of considerable importance in relation to the overall safe operation of the site." The Nuclear Installations Inspectorate is investigating possible breaches of license conditions or of the Health and Safety at Work Act and is requiring BNFL to measure the extent of the contamination and its flow rate and is asking BNFL to devise a way to contain or remove the material.

New study predicts widespread unemployment from micro-processors: A new study analysing the introduction of micro-processor technology in telecommunications, office work and journalism predicts up to 40% job loss in these areas and an overall increase in unemployment in the United Kingdom from its present level of 1.5 million to 3.5 million, rising to 5 million by the mid-1990s. The study, by Counter Information Services, a London-based collective of

journalists, is sharply critical of government claims that jobs will be created in the specific areas of silicon chip production, computer programming and service industries.

The report argues that silicon chip manufacture is capital intensive and is likely to be sited in the cheap labour areas of south-east Asia and the southern US, that computer programming is itself being automated through the introduction of modular programming techniques and that the service industries, transport and communications, insurance and banking, and the distributive trades are among the first to be hit by the new technology. In addition CIS predicts that profits from microprocessors will be re-invested in the financial sector rather than in manufacturing thus making job creation less likely. (*CIS Report, The New Technology*, 9 Poland Street, London W1. 75p.)

Pollution prevention pays:

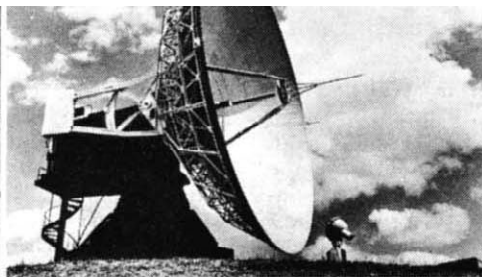
An analysis by a British chemical engineer has led to the introduction of equipment that has produced sizeable profits from pollution prevention. Dr Michael G. Royston of the Centre for Education in Management in Geneva is the winner of a £1,500 CONOCO award for his work on environmental problems facing industry. Royston looks at pollution



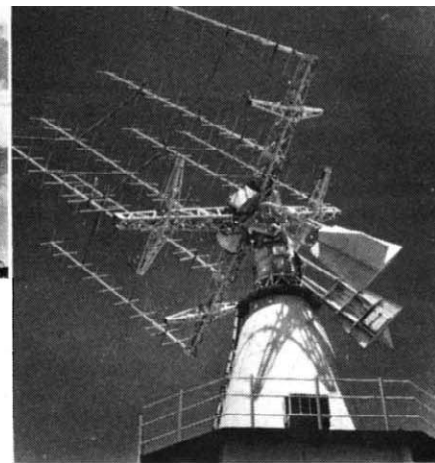
"Our factory doesn't actually make anything... we make more just producing waste!"

in terms of waste. He has been instrumental in persuading industry to consider pollution prevention as the recovery of waste materials which are useable and/or saleable. The Minnesota Mining and Manufacturing Company introduced the programme into its subsidiaries in 15 countries resulting in the elimination of 70,000 tons of air pollutants and 500 million gallons of waste waters with an annual savings to the company of £6 million. In the UK, Dow Chemicals saved £3 million in materials after three years and North British Distilleries installed effluent evaporators at a cost of £600,000 and sold the dried waste as a high protein animal feed for over £1 million in the first year. Royston sees industry, society and their common environment as a "seamless web" and calls for industrial managers to exercise their responsibilities accordingly. (*M. G. Royston, Pollution Prevention Pays*, Pergamon Press, Oxford.)

German psychologists issue study of Berufsverbote: The German Society for Behaviour Therapy (DGVT), the largest professional organisation for psychotherapy in West Germany with 6,000 members, has issued an official report on the effects of the West German government's policy of Berufsverbote (ban on professional employment). The DGVT estimates that there are "several thousand victims in different vocational fields" and that over one million people have been subjected to "routine" political screening since 28 January 1972, the date of the Minister-President's decree. The Berufsverbote in West Germany has led to firings of specific individuals, vocational discrimination and "psychological effects on non-victims as well as on victimised persons". The report criticises violations of confidentiality in psychotherapy and "hair raising" conditions in institutional psychiatry. Respondents to a questionnaire stated that they feared having communist friends, were "prudent" about signing petitions and avoided shopping in "leftish" bookshops. (*Berufsverbote in the Federal Republic of Germany*. Geschäftsstelle der DGVT, Postfach 1343, D-7400, Tübingen.)



India's space men head for self-sufficiency



Pictures (left to right): Sriharikotta launching range; earth station at the Space Application Centre; Satellite tracking antenna.

DURING his visit to the Soviet Union next month, Prime Minister Desai should witness the launching of India's space satellite from a Soviet launch pad. The USSR launched India's first satellite in April 1975 under this co-operation agreement.

While India's first satellite, Aryabhata, had a scientific payload, the 425-kg second satellite—Satellite for Earth Observations (SEO)—will have a remote sensing payload of two TV cameras and a microwave radiometer. SEO will help engineers of the Indian Space Research Organisation (ISRO) to conduct experiments aimed ultimately at developing operational remote sensing systems. SEO will be able to collect data about forests, soil types and snow cover and will be useful for preparing land use maps, determining how much forest has disappeared and predicting summer water flows in the northern rivers by estimating the snow cover in the Himalayas.

India's space researchers hope eventually to build and launch their own satellites. ISRO consists of four major space research and development centres, the most important of which is the Vikram Sarabhai Space Centre (VSSC) at Thumba near the southern tip of India. VSSC employs more than 4,500 people and concentrates on building and designing rockets.

The Sriharikotta rocket launching range is on a small island off the south-east coast of India near Madras. The satellite development and construction centre is at Bangalore in the southern state of Karnataka, while the Space Applications Centre is at Ahmedabad in western India.

SAC's research is aimed at developing space technology that complements major national development programmes. For example, SAC was responsible for the ambitious Satellite Instructional Television Experiment under which the US satellite ATS-6 was used to beam educational television programmes directly to 2,400 villages.

ISRO also intends to develop opera-

tional remote sensing systems which could help food management, particularly during a drought. If harvest yield can be predicted a few weeks in advance, state procurement and distribution of grain will be more efficient. Remote sensing could also help in surveillance of crop pest and disease and in groundwater and mineral exploration.

Towards the end of this year or early next year, India will be launching another satellite, Rohini satellite-1 (RS-1), this time on a rocket designed and built by its own engineers at Thumba and launched from Sriharikotta. The Indian rocket, currently nicknamed SLV-3, is similar to the US Scout rocket. SLV-3 will use solid propellants in all its four stages. It will put the small 40 kg RS-1 into an elliptical orbit with a perigee of 300 km and an apogee of 830 km. The plants producing the solid propellants for SLV-3 have been designed by Indian chemical technologists. Some 30 Indian industries are involved in making components for SLV-3. Once SLV-3 is completed, India will launch a series of Rohini satellites.

The SLV-3 and the Rohini series are, however, an intermediate goal in ISRO's plans to build and launch its own communications satellites by the late 1980s. India currently has a project with the European Space Agency called APPLE—the Ariane Passenger Payload Experiment—under which an experimental flight of the ESA's launcher will put a small India-built communications satellite into an orbit near the geosynchronous orbit. With the help of a small rocket, ISRO will then boost the satellite into a geosynchronous orbit and use it for minor communications experiments. This flight, scheduled from mid-1980, should help India gain valuable experience. Construction of APPLE is now in an advanced stage.

Meanwhile, because of growing telecommunications needs, particularly between its major cities, India has already ordered two operational com-

munications satellites from the Ford Aerospace Communications Corporation in the US. The first will be launched by NASA in March 1981. An important factor behind these orders was India's fear that the geosynchronous orbit over India could get over-crowded by Soviet, Japanese, INTELSAT and even Arab satellites. India therefore had to get a parking place and reserve broadcasting frequencies for its own satellites. To study and build the type of communications hardware required on the ground for a communications satellite, India has been conducting a two-year experiment called the Satellite Telecommunications Experiment Project (STEP) with the help of the Franco-German satellite, Symphonie.

Apart from the Soviet Union, France is also emerging as an important space ally of India. France is helping India to acquire know-how for building high-thrust liquid rockets that will be needed for launching communications satellites. The French, in turn hope that the APPLE project will create enough interest for India to become a customer of their Ariane vehicle for launching future Indian communications satellites.

The Indian space programme might appear ambitious and costly, but being based in a developing country keeps costs down. Much of the cost of advanced technology research goes in salaries for highly trained staff. Skilled technicians, mathematicians and engineers are available in India often at less than a fifth of Western salaries.

In the last ten years, ISRO has spent about Rs2,500 million (£150 million), which is tiny compared with Western space programmes. This entire expenditure, however, will have to be justified by its contribution to India's national development programme without becoming an unnecessary scientific status symbol.

Anil Agarwal

Joseph Hanlon reports on two more aspects of science in Ghana

Even poor countries need luxuries

Does Ghana need a nuclear reactor? Nkrumah thought so, and with help from the Soviet Union set up the Nuclear Research Establishment at Kwabenya, near Accra. The first military government thought not, and ran it down. The second military government disagreed, and is now building it up again—with help from Britain, the UN, and other donors. The establishment is now the largest—and most controversial—science project in Ghana. It lacks only one thing—a reactor.

Critics dismiss the project as a colossal waste of money that could be better spent on other areas of science with more direct impact on development. Even supporters concede that little of practical value has been achieved—yet. But they argue that developing countries need centres of excellence.

"There must be a national consciousness that what we have now is not what we should have. There is a need in developing countries to have centres of development which serve as indicators of what can be", declared Professor J. E. O. Lindsay, secretary to the Ghana Atomic Energy Commission (GAEC). "In each rural area, we should build a water closet at one point. I want people to strive and aspire for this. People should know that a pit latrine is primitive.

"I firmly believe that this establishment will serve as such a centre of development in science and technology. Experience has shown that those countries which have embarked on nuclear programmes have achieved spin-offs. We have to lift ourselves out of the technological doldrums", he said.

Nkrumah first organised the project in the early 1960s "when the big powers were testing nuclear weapons. France had a test site in Mali. The fallout posed a health hazard to nearby countries such as Ghana", Lindsay noted. Nkrumah also saw the establishment as the centrepiece of a science city.

It cost about £4 million to build—half a loan from the USSR that still must be repaid. All the equipment, down to the telephones was Russian. In February 1966, when Nkrumah was overthrown, the project was less than a year from completion. All that remained was to install the Russian swimming pool reactor. But the new military government threw out the Soviet technicians and brought the project to a halt.

Sir John Cockcroft was called in from Britain to advise on the project later that year. He concluded: "Taking into account the present state of science and technology development

and potentialities of Ghana, the scarcity of high level manpower, and the need for more financial support for research in agriculture, health, and many other fields contributing directly to the economic development of Ghana, I consider that the scientific and technological case for proceeding with the reactor project is extremely weak." He proposed that no reactor be installed, and that the establishment be run down to a small radioisotope centre. This was done. The GAEC was dissolved and practically all senior staff transferred.

But the head of the second military government, General Ignatius Acheampong, expressed interest in the project soon after he took over in January 1972. In May 1972 a committee was set up to review the project.

The committee noted that considerable money had already been sunk into the project. It concluded that "on the whole the successful execution of the aspects of the programme relating to agriculture and pest control alone will seem to justify the investment in completing the reactor; also, Ghana would then have established a strong programme in a field bound to become more important in many respects in the decade ahead."



Professor Lindsay: setting an example

The priority attached to the revived GAEC can be judged from its budget—nearly 4 million cedis last year, including 1.5 million cedis in foreign exchange (£1.7 million and £650 000 at the exchange rate then). By comparison, the entire budget of the Council for Scientific and Industrial Research (CSIR) was only 21 million cedis, and it received no foreign exchange. Senior scientific staff at the nuclear establishment now number close to 100.

Much of the past few years has been spent rehabilitating the buildings and equipment and rebuilding the staff. The project is now in much the same

position as 13 years ago—complete with all but reactor. It was decided that the swimming pool design is now obsolete, so a new type of reactor will have to be installed in any case. Negotiations are still under way—with Germany, Britain, and the US—to find someone who will supply a reactor and absorb some of the costs. The USSR is not now involved, according to informed sources, in part because of outstanding debts.

A major setback for the project was a fire in December in a cold store in which the most valuable equipment was stored.

Much of the research at the establishment is in traditional nuclear areas such as irradiation to preserve food, mutation to improve crop plants, and radiation-linked pest control, particularly sterile male techniques involving tsetse flies and anopheles mosquitoes. Lindsay proposes that all cocoa—Ghana's primary export—should be irradiated with Cobalt-60 to retard mould; this would require large investment in nuclear facilities (perhaps £500 000) and considerably increased transporting of cocoa.

The Nuclear Research Establishment is collaborating with the Centre for Scientific Research into Plant Medicine, primarily through the use of radioisotopes. For example, to test an anti-diabetic herb, rats are injected with radio-carbon-labelled glucose and then fed the drug. If the drug is successful, the glucose is metabolised and the radioactive carbon is found in the expired carbon dioxide.

Critics in the Ghanaian scientific community point out that much of the work done at the nuclear establishment is there only because it involves quite low level isotopes. In developed countries, such work is commonly done in ordinary labs, and could easily be done in CSIR labs, the plant medicine centre, and so on. Indeed, similar work is already done at Korle-Bu Hospital.

Further, they point out that most of the research work has already been tried unsuccessfully elsewhere, or can be done much more cheaply by non-nuclear methods.

The Nuclear Research Establishment faces the same problems as other scientific units in Ghana. It has electricity blackouts—some times for several days. Often it cannot get supplies; it is said that there have been no radiation film badges for 18 months.

Nevertheless, there is a feeling that it is too late to turn back—too much has been invested. Also, many feel that despite what Cockcroft said, this money would not be available for other scientific projects if the GAEC were not there. For example, the International Atomic Energy Agency continues to give large amounts of money—

it contributed \$194 000 last year—and the British government seems particularly anxious to help this project.

The fate of the Atomic Energy Commission really rests with the civilian government to be elected next month.

Stopping the desert's march

As the Sahelian drought continued and the desert moved south, Ghana looked nervously at its Upper Region. Understanding of desertification was—and still is—very poor although it does appear that deserts are often manmade.

So Ghana in 1975 set up the North-east Savannah Research Project, centred on Bawku in the Upper Region. As if to prove the wisdom of the decision and to give the scientists an unexpected experiment, the area was hit a year later by its own drought, causing substantial crop failures and requiring international food aid.

One reason that desertification is not well understood is that it results from a complex mix of social and agricultural patterns, as well as climate, soil, and water. Changes in any one affect the others. The Savannah project involves agronomists, botanists, soil scientists, water engineers, meteorologists, sociologists, anthropologists, and economists.

The project's final report will not be completed until later this year, and there are complaints that bureaucracy has stifled research. Nevertheless, the research has already challenged some conventional wisdom.

The population of the area has doubled in the last 25 years. There is no longer enough land for traditional agricultural practices, and there are clear signs of land degradation; radical farming changes are necessary if people are to be fed without destroying the land.

To try to work out such changes, the Savannah project was set up with \$500 000 from the US Agency for International Development (USAID) and 500 000 cedis from the Ghana government. Participants include the Council for Scientific and Industrial Research (CSIR) and several of its research institutes, the University of Science and Technology (UST), the University of Ghana, and the University of Arizona in the US.

Although Ghana's Upper Region contains one-tenth of the country's population, little attention was ever paid to it—either before or after independence. So the first problem was simply to collect data—on settlement patterns, crops, rainfall, etc.

Several parties are committed to reopening all of Nkrumah's prestige projects closed down by the first military government.

Whatever happens, the most common view was expressed by one physi-

cist: "The reactor is just political—there is no sound scientific programme to justify it. But if the politicians want it, then scientists should make the best use of it. And even poor countries need luxuries." □



North-east Ghana: a typical compound during the dry season

A study by the UST Land Administration Research Centre charted both the problems and local knowledge. Soil erosion, as expected, is the most serious problem, affecting three-quarters of the farmers studied. But the farmers are responding—more than half have introduced contour ploughing. Land pressure due to rising population means that land is no longer left fallow for long periods—but the farmers realise that they must take steps to maintain fertility and are knowledgeable about manure and fertilisers.

One significant finding was that the standard method of improving yield—using chemical fertilisers—simply will not work. This is now being studied in the area by a massive World Bank and UK project. But the study found that the value of the increased yield is less than the cost of the fertiliser. It also found that one village, Pusiga, had yields up to six times higher than surrounding villages—using basically traditional methods. So there is clearly room for substantial improvement elsewhere.

Other significant research has been done on the annual burning of bush and crop stubble—traditionally blamed as the prime cause of land degradation. Colonial and post-colonial governments through Africa have tried to stop burning—unsuccessfully. Although few studies have been done, ecologists have assumed that burning is almost all bad, reducing the mineral content of the soil and exposing it to wind erosion.

A joint burning study by scientists from the University of Ghana botany department and the Forest Products Research Institute has countered some of the conventional wisdom. Burning, for example, has virtually no effect on soil nutrients. And it has surprising benefits (which the farmers knew all along). If timed correctly, burning actually increases the amount of grass, providing more food for cattle, and it encourages growth of useful grasses.

This and other research work means that the Savannah project is likely to meet two of its three objectives, as spelled out by project secretary Dr Robert Dodoo. They are the identification of factors involved in savannah land degradation, and the identification of actions to be taken to reverse this trend.

But the project seems to have failed in what Dodoo cites as the first objective: "To strengthen the capability of the Ghanaian scientific community to conduct interdisciplinary research which contributes directly" to development. The Savannah project was a direct result of a CSIR/USAID attempt to find a research project which would prove coordinated work possible.

Unfortunately, all of the participants I spoke with complained of a lack of coordination and cooperation. For example, satellite pictures were not passed on from the remote sensing group to others, such as the water management group, which wanted them. Various study groups were not permitted to circulate preliminary reports to each other, even though this would have been highly useful. And contacts between researchers on this project and other research and development projects in the Upper Region were limited.

It is too early to tell if the North-east Savannah Research Project will meet its overall goal: to "improve the well-being of rural people in northeast Ghana". But, despite coordination difficulties, it is already clear that it will make an international scientific contribution. As Nii Ayibotele, chief research officer of the Water Research Institute and a savannah project team leader, explained: "This is one piece of an international data jigsaw that should lead to a theoretical understanding of desertification". □

Dr Hanlon is a science journalist specialising in Third World Affairs.

A tribute to a man behind a movement

Joseph Rotblat recalls the part played by Cyrus Eaton—who died recently aged 95—in establishing the Pugwash Movement

CYRUS Stephen Eaton was a unique blend of tycoon and philosopher, shrewd businessman and altruist; he believed in capitalism and espoused closer relations with communist regimes; he controlled a billion-dollar industrial empire but took delight in the company of scholars. Unlike Carnegie and Rockefeller he did not endow foundations but he brought Pugwash to life.

Pugwash, a fishing village on the Northumberland Strait in Nova Scotia, is where Cyrus Eaton was born. For the first 70 years of his life hardly anybody outside had ever heard of it, and when the name became familiar it was that of "Captain Pugwash", a character in comic strips and children's books. But now Pugwash, with its proper definition, has an entry in the Oxford Dictionary.

After studying for the Baptist Ministry and taking a degree at McMaster University, Eaton went into business, first under Rockefeller and later on his own. He very quickly amassed a huge fortune, by branching out into many industries and founding new enterprises. His empire included gas, electricity, coal, iron mines, steel, rubber, railways, shipping and banking.

Mr Eaton also indulged in various hobbies, including hunting and prize bull breeding. But amidst all this he found time to study philosophy; he told Bertrand Russell that he read all his books again and again. Finally he decided to give these pursuits a more concrete form. In 1955 he set up an educational trust, and began to invite to Pugwash, which he used as a summer residence, scholars and educators to relax, exchange views and sharpen their own thinking. But although these Pugwash encounters went on for a long time, they were eclipsed by the real Pugwash which was initiated by Russell.

Alarmed by the increasing pace of the nuclear arms race, Russell, Einstein and nine other scientists issued in July 1955, in London, a manifesto (Einstein signed it just before he died) drawing attention to the new peril to mankind, and calling on scientists to assemble in a conference to assess the situation and

devise means to avert a catastrophe. As soon as he heard of the Manifesto Cyrus Eaton wrote to Russell offering to finance the conference anonymously and suggesting Pugwash as the venue.

At that time it was planned to hold the conference in India where Nehru had offered hospitality. But there was still the problem of travelling expenses, and Russell wrote to Eaton in 1956 asking him to assist in this. Eaton's reply was a polite refusal and a reminder that the original offer was for a meeting in Pugwash. As it happened, the plan for India had to be abandoned in any case, due to the Suez crisis. Mr Eaton's offer was taken up and the conference finally assembled in July 1957—in Pugwash.

It turned out to be an historic event. It was the first time—and at the height of the cold war—that scientists from East and West (including the US, USSR, UK and China) met to discuss matters which statesmen had found intractable. It was a real meeting of minds and opened a new channel of communications which the governments on both sides subsequently found most useful. Thus, a new movement of scientists was born, the Pugwash Movement.

The success of the Conference was to a large degree due to Cyrus Eaton and his friend Mrs Anne Jones, who

acted as hostess. They created the congenial atmosphere so essential for a free and frank exchange of views. But if they influenced the Conference, it had a profound influence on them.

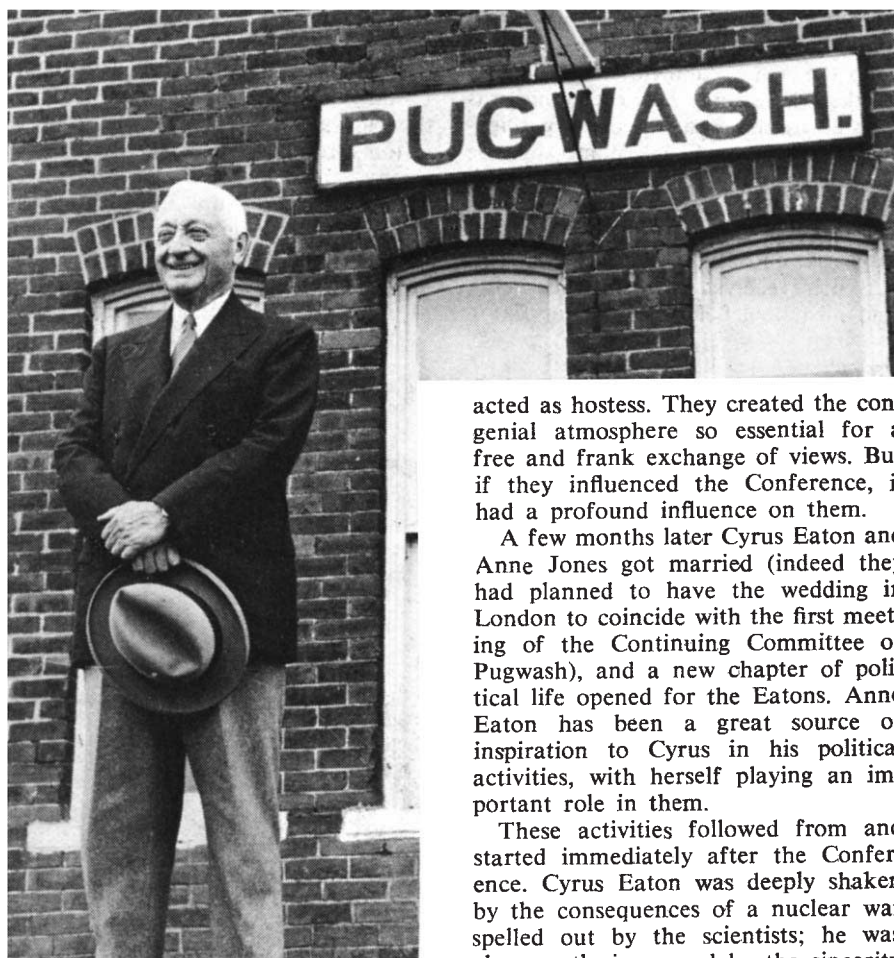
A few months later Cyrus Eaton and Anne Jones got married (indeed they had planned to have the wedding in London to coincide with the first meeting of the Continuing Committee of Pugwash), and a new chapter of political life opened for the Eatons. Anne Eaton has been a great source of inspiration to Cyrus in his political activities, with herself playing an important role in them.

These activities followed from and started immediately after the Conference. Cyrus Eaton was deeply shaken by the consequences of a nuclear war spelled out by the scientists; he was also greatly impressed by the sincerity of the Russian participants and their expressed desire for peace. He therefore launched a campaign, widely publicised, advocating détente with the Soviet Union and the recognition of China. In all this he was years ahead of political thinking in the West.

Predictably, Eaton incurred the wrath of the hawks, and was savagely attacked by the Committee of Un-American Activities. On the other hand, he was *persona grata* in the Soviet Union which gave him its official approval by the award of the Lenin Peace Prize in 1960. He became friendly with Khrushchev, who presented him with the famous troika of horses, and Eaton reciprocated with a gift of his prize steers. Eaton's strenuous efforts towards détente and nuclear disarmament were later extended to the anti-Vietnam war campaign.

These objectives were shared by the Pugwash Movement, but the approach to them differed radically. Pugwash shuns publicity: its meetings are private and their main purpose is to provide a forum for the exchange of ideas which are transmitted to decision makers by the participants. Cyrus Eaton had at his disposal an immense public relations organisation.

Inevitably there was a clash; some American scientists felt so embarrassed



by Eaton's statements which he linked with Pugwash, that they wanted to drop the name Pugwash from the title of the Movement. However, this was resisted by the great majority of scientists, and the official name of the Movement remained Pugwash Conferences on Science and World Affairs.

Because of Eaton's direct link with Pugwash and the publicity which his activities received, there is a general, and mistaken, belief that Cyrus Eaton

was the founder of the Pugwash Movement. The real founder was Bertrand Russell; it was at his call, and not Eaton's, that the scientists gathered in the Pugwash Conferences. Nevertheless Eaton's role was crucial; without his timely and generous assistance the Movement might not have taken off.

For this reason Pugwashites have not only deep gratitude but special affection for Cyrus Eaton. Although after the first few conferences he no

longer made direct financial contributions to them, he a Mrs Eaton attended a number of conferences in the role of guests, and were always warmly greeted as friends and members of the family. The whole Pugwash Movement will deeply mourn the passing of a great man, a valiant fighter for peace. □

Professor Rotblat was Secretary-General of the Pugwash Conference 1957-73 and is still closely involved with the movement.

The explosive issue of nuclear power—Sir John Hill

Non-proliferation, it has been said, is like motherhood. Everybody is for it. But, unlike motherhood, not everybody agrees on how to achieve it. In this article, I hope to explore the nature and causes of nuclear weapons proliferation and in particular the balance between technical and political factors in the fight against proliferation.

In public discussions of the proliferation problem, too much attention may have been given to the question of plutonium and insufficient paid to the production of other fissile materials which could prove of equal, or even greater attraction to the would-be proliferator. Additionally, there is a considerable range of techniques available to the state seeking a supply of ^{239}Pu . High on the list would be dedicated production reactors and certain types of research reactor. Lower on the list, in my view would come accelerators and power reactors: accelerators, because no such machine has yet been built of sufficient power to be able to produce fissile material in significant quantity; power reactors, simply because they would provide a disproportionately expensive way of obtaining weapons quantities of fissile material.

Furthermore the possibility of producing nuclear materials does not of itself act as one of the driving forces of proliferation. Over the past 30 years fissile materials have been isolated and processed in abundance, without more than a handful of nations having gone down the road towards a nuclear weapons capability. The Non-Proliferation Treaty of 1968 has provided over a hundred states around the globe with the means of registering in a clear and unambiguous manner their intention to abstain from nuclear weapons development. South America is well on the way to the establishment of the militarily denuclearised zone provided for by the Treaty of Tlatelolco. There are further treaties setting constraints on nuclear weapons deployment in specified areas. The element of political will, witnessed by this reluctance by most countries to encourage the spread



The chairman of the United Kingdom Atomic Energy Authority (above) argues that the cause of non-proliferation is not served by attacking nuclear energy as a whole

of nuclear weapons is, I submit, of the first importance in assessing the causes of and remedies for proliferation.

The potential of diversion of civil nuclear materials to military applications has always been recognised by the civil nuclear industry. What has also been known is that technical means could be devised to render the diversion of nuclear material extremely difficult, indeed effectively impossible without alerting the world at large. It is my belief that this, coupled with the inherent unsuitability of the civil nuclear fuel cycle for military use, will conspire to make it perceptibly a far less suitable route towards weapons manufacture than any dedicated facility.

International safeguards are the cornerstone of measures to demonstrate that civil facilities are not being used for weapons purposes. We can and should seek to ensure their enforcement by measures which would make it as difficult and as time-consuming to divert fissile material from its legitimate use as to build a completely separate weapons capability.

The criteria one could sensibly adopt in evaluating the proliferation-resistance of fuel-cycles have been looked at in the context of the US NASAP programme and are currently being examined as part of the International Nuclear Fuel Cycle Evaluation programme (INFCE).

In each case the criteria involve the assessment of technical choices, on matters ranging from the established machinery of safeguards systems, to the choice between fuel cycles themselves. (It seems likely to me that on detailed consideration the plutonium-based fuel cycle promises to be as effective in resisting proliferation as any other fuel cycle.) But although such technical choices are of great importance they cannot be considered in isolation from the underlying institutional features. To take one familiar example, it is often asked whether a fuel cycle involving reprocessing is a better or a worse thing from the proliferation standpoint than one simply involving the storage of spent fuel. This, I suggest, is the wrong way to ask the question. There is, in my view, nothing in the nature either of reprocessing or of spent fuel storage that is of itself conducive to weapons proliferation. The situation that we find in the world at present is a growing number of countries who each have only a small nuclear component in their generating systems. For such countries there is little attraction economically speaking in installing their own reprocessing capacity. Their most attractive solution is to purchase reprocessing services from an existing plant such as Windscale. As an alternative, they could participate perhaps together with suppliers, in the establishment of some kind of multinational institution which would provide fuel-cycle services to countries with a legitimate end-use for the fissile materials. In addition, there is a strong case for seeking international agreement on a regime for handling separated plutonium. A system for the international storage of plutonium, of the kind under discussion with the

IAEA, could make a valuable contribution to providing increased confidence that the movement and use of plutonium are internationally known and carefully monitored. Complementary multinational or international arrangements could also exist for the storage of spent fuel which would again prove economically attractive to their users. There is no doubt, in my mind, that arrangements of this kind, where plutonium would be returned to customer states only under safeguards, would minimise any proliferation risk from spent fuel storage or reprocessing.

Were, on the other hand, arrangements of this kind not to be available then countries lacking fuel services would be obliged in the short term to extend their spent fuel storage capacity: in the longer term, they might, for reasons of security of energy supply, find it increasingly attractive to provide themselves with an indigenous reprocessing capability. Such developments, while they of course would by no means necessarily lead to the decision to separate fissile materials for military purposes, nevertheless put fewer barriers in the way of weapons proliferation than the kinds of multilateral arrangement outlined above.

What all of this shows is that what appear to be purely technical questions turn out on closer analysis to have a large political dimension, and while we can and should submit these technical issues to searching scrutiny, we should not forget that this is only half the story. Indeed at the end of the day it is the political measures which will, in my view, turn out to be the really important issue.

A breathing space before the switch to plutonium

What then should be our strategy with respect to the future of nuclear power? Would the cause of non-proliferation best be served by cutting loose totally from the nuclear option? From what I have said, it is, I hope, clear that the answer to this must be no. The least that could be said of the strategy sometimes advocated of cutting back on nuclear power is that it would prove totally irrelevant to the task at hand. Indeed I might go further, and say that such a cut-back would if anything jeopardise our non-proliferation objectives, since it would prove unacceptable to those nations on whose cooperation we rely in our attempt to ensure that non-proliferation remains possible.

What of plutonium-based fuel cycles? For the remainder of this century, there is no doubt that the world's nuclear power capacity will

continue to be made up in the main of uranium-fuelled thermal reactors and that the extensive commercial use of plutonium as a nuclear fuel in any part of the world is still some years off. This provides us with a breathing-space to explore ways in which we can help to maximise the proliferation-resistance of plutonium-based fuel cycles, in preparation for the time that their commercial development becomes desirable. It is, I think, now generally accepted that the reprocessing of nuclear fuel and the use of plutonium will eventually become a necessity for many of the countries which have a nuclear power programme. How soon this necessity will arise will depend on features which will vary considerably from country to country.

One important objective of INFCE will be to enable countries to identify their optimal course of action regarding the introduction of plutonium-fuelled reactors given their own position in respect of energy supply and demand and other factors. For most countries the economic case for the thermal recycle of plutonium will probably at most be marginal. The fast reactor fuel cycle by contrast will, I feel certain, come eventually to be seen by all countries, with a major nuclear component in their generating systems, as offering a significant economic advantage and will eventually supersede the thermal reactor cycle as the mainstay of the world's nuclear generating capacity. And so long as we have the right institutional features, there is in my opinion no reason why the use of plutonium as a fuel should render impracticable the task of protecting fissile materials.

Again it might be asked, would our best strategy be to retain all of our civil nuclear technology including fast reactors, but do all we can to limit the access of any further nations to this technology? My conviction is that this would be a profound mistake. It was clear to the nations which negotiated the Non-Proliferation Treaty that if the vast majority of the countries of the world were to be persuaded to give up a defence option of great significance, then this could not be done without an adequate *quid pro quo* being offered. The incentive that was offered in the NPT, and it was in my opinion the correct one, was assistance from the nations with the greatest nuclear expertise to the non-nuclear weapons states in developing the peaceful applications of nuclear energy. This is made explicit in Article IV.2 of the Treaty. The idea that the safeguarding of the nuclear fuel cycle should go hand in hand with the promotion of the peaceful uses of atomic energy was already implicit in the statute of the IAEA,

which from its inception was given the dual task of promoting civil nuclear power and ensuring safeguards.

What I have said should not be taken as endorsing the unrestricted spread of nuclear facilities to countries that can make no real use of them. What I do however most strongly believe is that, where nations are in a position to benefit from the peaceful uses of nuclear energy, then the world is not only entitled but even duty-bound to assist them in their development. If the developed world is not prepared to share its use of nuclear technology, there is every likelihood that countries refused access to the use of this technology will feel themselves impelled to compensate for this through indigenous development.

Protecting the security of non-nuclear nations

More than anything else, what guides the choices of nations faced with the question of whether or not to develop nuclear weapons, is their perception of their national security and of the reliance they can place upon the international community to protect them. One finds for instance in the statements made by a number of nations at the time that they signed the NPT in 1968, references to the importance that these nations attach to the promise of intervention by the United Nations Security Council in the event of their being threatened with nuclear attack. If we are to be successful in the struggle against nuclear weapons proliferation, one important objective must be to ensure that the non-nuclear weapons states do not regard their security as being endangered by the non-proliferation regime. This may involve the international community in concerted political action if the occasion so demands. It also involves the protection of other vital interests including, I believe, assurance of adequate energy supplies to all nations around the world, and it is from this point of view that the development of civil nuclear technology may be important in fighting proliferation.

The possibility of nuclear power and possibility of nuclear weapons have both been with us now for many years. To a very considerable extent the development of these two technologies has been separate. But in the last analysis there is no such thing as the civil atom or the military atom. An atom is a neutral thing and technology is a neutral thing. It is one of the most important moral and political duties incumbent upon mankind as custodians of this earth to ensure that the technology of nuclear energy is one that is turned to good not evil. □

correspondence

World energy organisation

SIR,—Mr Greenhalgh (26 April, page 776) takes me to task for views I presented in a lecture and briefly reported in *Nature* (1 March, page 4). If *Nature* had given as much space to the report as to Mr Greenhalgh's letter, he would have found many of his arguments answered.

Mr Greenhalgh says there is a need for a greater concentration and coordination of the various projects of non-nuclear energy sources. This is exactly what I gave as one of the reasons for setting up a World Energy Organisation. At present there is no body in existence to carry out this task, and without a permanent institution the sporadic efforts will be highly inefficient, resulting in much duplication in some areas and gaps in others. But the proposed World Energy Organisation, as I envisage it, would do much more than promote research. It would advise countries of the type of energy most suitable for them—including nuclear if deemed necessary—as well as provide funds for the implementation of the advice. Considering that energy is the life-blood of modern society, a World Energy Organisation is essential. I am convinced that it would have been set up long ago if it were not for the International Atomic Energy Agency. It was a great mistake that the only truly international effort on energy should be concerned solely with one form of it; diversification of energy sources is vital for the world.

IAEA is doing a good job in fulfilling the objectives for which it was set up and I am not calling for its abolition, but it would be unwise to enlarge its terms of reference to include other energy sources. I am also not very happy about IAEA having two largely contradictory tasks: promoting nuclear energy and safeguarding against its misuse. I should like to see the latter be made part of the authority needed to implement the internationalisation of the sensitive parts of the nuclear fuel cycle, which I also advocated in my lecture.

This brings me to the problem of proliferation of nuclear weapons. Mr Greenhalgh is Honorary Secretary of APG (A Power for Good), an action group to promote nuclear energy, and is duty bound to minimise the danger of proliferation arising from the widespread use of nuclear energy, but his arguments carry no conviction. A nation intending to acquire nuclear weapons is not likely to make the preparations openly or in ways that can be easily detected. On the other hand, starting a nuclear power programme will enable it to acquire legitimately the technology and the materials for nuclear weapons. Moreover, this would also be the cheaper way because reactor-exporting countries will be only too eager to offer generous, long-term loans; in the long run this would be disastrous to the recipient country but it would hardly deter a regime which is bent on getting nuclear weapons.

The argument about the oil crisis is completely irrelevant. How can nuclear energy at its present rate of growth, become a substitute for oil when it runs

out, or even have a significant effect on the oil crisis? Of course, there may be a confrontation over oil; there is also a multitude of other causes of conflict which may lead to war, but every sensible man can see that the consequences of any war would be far more disastrous if the combatants used nuclear weapons.

Finally, I don't need Mr Greenhalgh to remind me that the greatest danger to mankind comes from the nuclear arsenals of the great powers. I realised that danger even before the day of Hiroshima, and ever since I endeavoured to do my utmost in the efforts to reduce it. But the hypocrisy in Mr Greenhalgh's argument is obvious: he draws attention to one source of danger in order to divert it from another source. Horizontal proliferation is a real issue, and its connection with nuclear power is generally recognised. If nuclear plants of the small nations did not present a potential danger, there would be no need for the NPT, the IAEA safeguards, the London Suppliers' Group, the INFCE, etc.

The avoidance of a nuclear holocaust is difficult enough with a few nuclear powers, but it would be hopeless if most nations became nuclear weapon states.

J. ROTBLAT

London, UK

Interpreting shadowed relief

SIR,—Everyone is familiar with photographs of the lunar surface showing craters, and with the fact that if such a picture is held upside down, flat topped mountains will be seen instead. The image is correctly interpreted as craters if the photographed light falls towards the viewer, and is wrongly interpreted as mountains if this light falls away from him. This reversal, sometimes called *pseudoscopic illusion*, seems to have been first described and correctly interpreted by Sir David Brewster (*Rep. Br. Ass. Advmt. Sci.* 30, pt 2, 7–8; 1860), is discussed by Helmholtz (*Handb. d. Physiologischen Optik*, 3rd ed, ch 30, Voss, Hamburg/Leipzig; 1910) and has received some attention in contemporary perception psychology (e.g. Rock, I., *An Introduction to Perception*, MacMillan, New York; 1975). If the orientation of a moon picture is midway between right and wrong the image will be found to switch between crater and mountain at irregular intervals, an effect sometimes described as *image salutation*. Faced with an 180° misoriented moon picture, no amount of conscious effort by the observer, telling himself he *ought* to see craters will make the image switch. This *orientation* cue can only be overridden by a very strong contrary cue, such as a familiar image which can only be interpreted in one way. Thus, a picture of a chair will be seen correctly even if its shadow falls away from the viewer. A crater is not such an object since the alternative, a flat topped mountain, is not an inconceivable moon feature. Stereoscopic vision may also overcome the orientation cue, as illustrated by R. L. Gregory (*The Intelligent Eye*, McGraw-Hill, New

York/London; 1970).

The electron microscopist examining shadowed replicas often finds himself in the frustrating situation of seeing the image in reverse relief, since there is normally no simple way of rotating a specimen grid without taking it out of the instrument and remounting it. Although the final photograph can always be correctly oriented, it is necessary to perceive relief correctly in order to understand what one is examining in the specimen itself. This is particularly critical for freeze-fracture biological replicas, in which the fracture plane can pass so as to yield both positive and negative relief. Often faced with this problem, I found a simple cue which can always be applied and is so powerful it invariably overcomes misorientation. It is simply to hold a small hand torch at the edge of, but within one's field of view, throwing light in the required direction. The light need not fall on the image; in fact the fluorescent screen of ordinary electron microscopes is so recessed this is impossible. Very little light is sufficient, so interference with dark adaptation can be minimal. Although a hand torch is unnecessary in the case of a photograph which can be oriented, it provides a good demonstration of the effectiveness of the cue. In particular, it will be found effective even in the presence of strong non-directional illumination.

Another form of this cue can be applied to lantern slides. It consists of a circular hole in the mask, just outside the border of the picture and in the sector from which light should come. For a 35mm slide, the hole should be about 1.5mm in diameter; for a 3½" x 4" slide, about 5mm in diameter. The audience is not told the purpose of the resulting spot of light just outside the projected image, but will unconsciously interpret it as a "sun" and see the relief correctly. However, if orientation is arbitrary, it is preferable for the slide to be so made that light falls from the upper sector, so that the light spot cue and the orientation reinforce one another.

There are many applications of this principle: pictures of the moon and planet surfaces; air photographs, particularly those dealing with archaeological traces; oblique radar mapping; light microscopy with Nomarski optics; electron microscopy of shadow cast replicas. In published reproduction it may be urged that if there are no cartographic or other scruples, orientation should always be such that the apparent light falls towards the viewer, who will then be saved the trouble of using an artificial cue. Instructions to authors in journals should request this. To avoid ambiguity, and particularly if photographs have to be printed in a misoriented direction, a small arrow should always be provided with the legend: *direction of falling light* (and not merely direction of light as this could be taken as pointing to the light source).

A. C. FABERGÉ

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news and views

First results from the Einstein Observatory

from A. C. Fabian

THE successful launch of HEAO-B in November 1978 opened a new era in X-ray astronomy. Renamed the Einstein Observatory, the satellite contains the first orbital X-ray telescopes to image cosmic X-ray sources. Four nested grazing incidence telescopes providing a resolution of a few seconds of arc over a field of a degree give a five hundredfold increase in sensitivity to source detection. The dynamic range available to X-ray astronomers now extends over seven decades from Sco X-1 ($\sim 10^{-7}$ erg cm $^{-2}$ s $^{-1}$ keV $^{-1}$) to the faintest Einstein source ($\sim 10^{-14}$ erg cm $^{-2}$ s $^{-1}$ keV $^{-1}$ at ~ 1 keV).

Preliminary results of the analysis of data streaming into the four groups involved in this experiment—at the Harvard-Smithsonian Center for Astrophysics (CFA), Massachusetts Institute of Technology, NASA Goddard Space Flight Center (GSFC) and Columbia University—were reported at the recent spring meeting of the American Physical Society in Washington DC. The HEAO-B principal investigator, R. Giacconi of CFA, showed images of the deepest survey exposures, which lasted several hours and revealed many faint sources. Approximately half of these sources appear to be quasars and the remainder are otherwise undistinguished stars. The X-ray properties of quasars are now seen to evolve in a manner similar to that found in radio and optical wavebands. Simple addition of the sources seen shows that quasars must contribute at least 20% of the extragalactic 1.0–3 keV X-ray background. The 2–50 keV HEAO-1 spectrum of the nearby quasar 3C273, discussed at the same meeting by D. Worrall of GSFC, is remarkably similar to that of the X-ray background over the same range. It seems a distinct possibility that a large fraction of the X-ray background may be explained by discrete sources if this trend continues.

Optical and radio astronomers are working in collaboration with the Einstein teams to study the fields

observed. Many of the new sources have yet to be identified, but at least one appears to be extended and may be a cluster of galaxies at a redshift ~ 1 . Most observations contain one or more sources, often in addition to the target source, and as Giacconi emphasised, the number of detected X-ray sources now stands at well over one thousand.

H. Tananbaum (CFA) discussed observations of clusters of galaxies, which are now resolved into clumps and peaks of emitting gas. The X-ray surface brightness of many of the rich clusters is strongly peaked around a central galaxy, whereas that in the less dense clusters breaks into knots that often coincide with individual galaxies. Extended emission from M87 underlies the Virgo cluster, which also shows diffuse clumps around M84, M86 and several other galaxies. The X-ray appearance of a cluster of galaxies may depend on the extent to which interstellar gas from individual galaxies has transferred into the general intracluster gas. Subsequent heating and cooling of that gas then decides its fate as the core of the cluster gradually relaxes, possibly leading to a buildup around a central massive galaxy. Several clusters around and beyond a redshift of 0.5 have been observed. These clusters were previously known from their association with radio galaxies, but appear to be normal in their X-ray luminosity.

The nearby radio, X- and gamma ray galaxy Centaurus A is found to contain several X-ray features apart from the bright variable nucleus. These include the inner radio lobes and an inner jet extending out to and aligning with a faint optical jet. X-ray emission from the inverse Compton scattering process in the radio lobes of Cen-A and similarly Cygnus A can now be used to measure the magnetic field strength and, it is hoped, refine models of the energy transport in these regions.

Our neighbour galaxy M31, which was barely detected by previous X-ray instruments, is now resolved into about fifty sources. Some trail spiral arms and many cluster in the central bulge.

One source coincides with the nucleus of the galaxy. Many of these sources are likely to be binary X-ray sources and supernova remnants with counterparts similar to those in our own galaxy. Studies of the source distribution in M31 should reveal much about these sources that is difficult to unravel locally.

The supernova remnants in the Large Magellanic Cloud (LMC) appear to be more X-ray luminous than those in our galaxy. D. Helfand of Columbia Astrophysics Laboratory showed several LMC remnants within the same field of view. A comparatively high interstellar medium density in the Clouds may be responsible, possibly causing supernova remnants there to enter the radiative phase after only ten thousand years or so. Several remnants in our Galaxy are well resolved into highly structured shells and other shapes. Some show features that correlate in detail with radio and optical pictures. The X-ray source associated with the Vela pulsar still fails to show pulses, unlike the Crab pulsar which stands out from the surrounding wisps. The front cover of this issue of *Nature* shows an X-ray image of the Cassiopeia A supernova remnant.

Images of the Orion nebula, the η Carina nebula and many other galactic regions contain relatively X-ray bright stars. O stars, late type stars, nebular variables and many others are now found to be X-ray sources. Diffuse emission from the region around η Car, which erupted over a century ago, is the backdrop to individual O stars and a Wolf-Rayet system. All of the well known galactic X-ray sources can of course be individually studied in finer detail than before. High resolution images of seven globular cluster X-ray sources pin their positions down to within a few arc seconds. The source in M15 is particularly close to the centre of the optical cluster core.

Spectroscopic results from the Einstein Observatory were presented by S. Holt (GSFC) and G. Clark (MIT). The Solid State Spectrometer revealed many lines in the emission from the supernova remnant Cas A.

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Holt demonstrated how the features of silicon, sulphur iron and several other elements dominate the emission below a few keV. Of particular interest is the possibility of measuring elemental abundances in the ejecta and testing the widely accepted view of

supernova enrichment for the interstellar medium. Line emission was also evident in the spectrum of the active galaxy 3C120 at the wavelength expected from its cosmological redshift.

It is clear that the results announced so far represent but the tip of the ice-

berg. Just about every class of astronomical object known outside the solar system is now found to emit X-rays. Blending these results with those at other wavelengths and with detailed theoretical models must lead to insights into the workings of the Universe. □

Dementia: an approaching epidemic

from Fred Plum

WE face now and in our future an increasingly prevalent neurological disease, which scientists can ill afford to ignore when choosing their research, for its effects will directly or indirectly influence the way we and our children live our lives. This is the problem of dementia, which already reaches almost epidemic proportions.*

Dementia is overwhelmingly a problem of man's declining years, and in the western world both the percentage and actual numbers of the susceptible population are increasing at an alarming rate. In the United States in 1950 about 8% of persons, a little over 12.3 million, were older than 65 years (Fig. 1). By 1978 the fraction had reached 11% and the involved population exceeded 22 million. Because of the relatively childless years of the depression and the Second World War these already large numbers are predicted to increase only moderately by the year 2000. But by the year 2030, when the postwar group of children reaches late adulthood, the US Census Bureau projects that somewhere between 17 and 20% of the US population will be older than 65 years, with the absolute number exceeding 51 million persons.

As we all know, and often too well from experience with our own friends and families, old age is frequently a neurologically impaired estate. Most of the 1.2 million patients occupying nursing homes in the US come from this age group. Furthermore, reasonably accurate health statistics indicate that dementia represents the major invalidating condition for at least half of these unfortunate persons. The present estimated annual cost of caring for patients with chronic dementia is a substantial 12 billion dollars. Future projections rise alarmingly: conservative predictions by the National Institute of Aging are that by the year 2030, the US will be spending 30 billion of 1978-value dollars for the chronic care of patients with dementia. One should recall that the total 1978 budget for the National Institutes of Health was 2.64 billion and that of

the Alcohol, Drug Abuse and Mental Health Administration was 1.13 billion (The exact NIH figure equals \$2,638,212,000. The ADAMHA budget of \$1,127,750,000 includes the appropriation for St Elizabeth's Hospital, Washington DC. These data as well as the other statistics were supplied by the Office of the Director, National Institute of Aging, NIH). Quick calculation makes it evident that the anticipated 2030 annual costs of care for persons with dementia amount to more than 8 times the amount we now spend on all medical and mental health research. By any standards, this represents an expanding epidemic of major proportions in terms of human suffering and societal expense. It also is an epidemic that can be prevented only by the successful attention of science and especially neurobiology to solving the problems that manifest themselves predominantly in the ageing brain.

Recent studies make it evident that most dementia reflects Alzheimer-senile disease, a primary cellular abnormality of the brain observed predominantly among persons in their later decades. (The biological aspects of Alzheimer's disease are well summarised in the proceedings of a recent NIH-sponsored workshop edited by R. Katzman, R. D. Terry and K. L. Bick, *Alzheimer's Disease; Senile Dementia and Related Disorders*, Raven, New York, 1978.) The condition does not predominantly reflect the effects of cerebral vascular disease as once was believed. Although widespread among the elderly, some persons altogether escape the disorder, suggesting that the abnormal cell changes reflect a specific inherited or acquired disease and not merely that the subject has outlived the foreordained life expectancy of the human race. Occasionally observed in subjects as young as 40 years or so, but rising thereafter to a peak incidence in persons between 70 and 75 years of age, Alzheimer-senile dementia causes more functional disability than all other causes of dementia combined (Table 1) (see Wells, *Stroke* 9, 1; 1978). Meticulous post mortem neuropathological studies demonstrate that the

brains of many non-demented elderly persons contain abnormalities that are qualitatively similar to those with Alzheimer-senile dementia but quantitatively very different. The principal pathological changes consist of atrophy of the fronto-temporal cortical gyri of the cerebral hemispheres, a reduction of cortical neurones, the presence of amyloid-containing plaques in the cerebral cortex, the development of neurofibrillary abnormalities in the cerebral cortex, the development of neurofibrillary abnormalities in the proteins of neurones and the appearance of a specific type of granulovacuolar degeneration of cells in the hippocampal cortex.

These quantitative neuropathological differences suggest that at least some of the anatomical alterations of dementia are an exaggeration or acceleration of the normal ageing process in brain rather than an independent disease. But other factors must operate as well. For example, certain external dementia-producing factors, such as the trauma of boxing (dementia pugilistica), and the chromosomal abnormality known as Down's syndrome, seem to intensify senile changes in the brain. It may be fruitful for the scientist to search for associated and potentially preventable conditions that abnormally accelerate the brain's

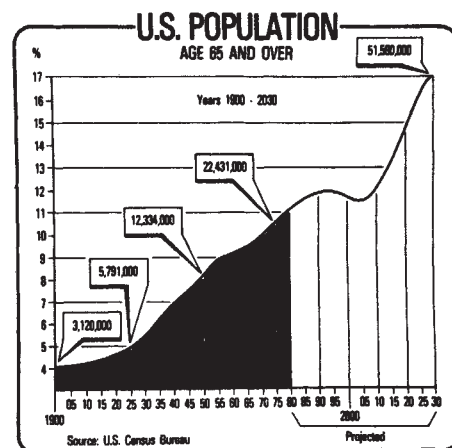


Fig. 1 Chart showing the percentage of the US population aged 65 years and older from 1900 to 1975, with predictions for 1980 to 2030.

*This is a truncated version of remarks given by Fred Plum during the Presidential Symposium at the Society for Neuroscience, St Louis, November 1978.

Table 1 Approximate frequency of diseases causing dementia

Alzheimer-senile dementia	50%
Cerebral vascular disease	8%
Nutritional, drugs, alcohol	10%
Brain tumours, trauma	6%
Huntington's Chorea	5%
All other causes	21%

natural rate of ageing or induce slow-ripening abnormalities that fail to exert their damage until life's later years.

Only a few clues guide neurobiological research in the Alzheimer-senile group of dementias. Many subjects with the disorder have a history of a similar illness in their direct antecedents, suggesting that at least some families inherit the susceptibility as an autosomal dominant trait. The presence of an immunoglobulin-containing core in the cortical plaques suggests the possibility of a yet ill defined immunological error. The abnormal neurofibrillary material in degenerating neurones is composed of protein filaments of a different size and composition from the protein subunits of normal neurofilaments. Recently several groups have demonstrated a reduction in the acetylcholine-synthesising enzyme, choline acetyl transferase, in the hippocampus and other areas of cerebral cortex of patients dying with Alzheimer-senile dementia. The finding gives hope that Alzheimer disease, like Parkinsonism, may turn out to be a specific neurochemical system degeneration, perhaps susceptible to at least temporary improvement with pharmacotherapy.

But even the discovery of a precise neurochemical abnormality in the most frequent dementing illness will leave us far from understanding the fundamental question. Nervous tissue suffers the inborn vulnerability that it cannot regenerate its damaged or dead nerve cells in a manner similar to that used by other organs in the body to replace their constituents. Why to a severe degree in many brains, and perhaps to some degree in nearly all, are the cell systems which transact the functions of memory, learning and judgement faced with the frequent risk of tangling up and dying before the cells of other organs or even before much of the rest of the brain? What is the genetic abnormality that predisposes or pre-terminates these neurones to a fore-shortened vitality? How can these premature degenerations be prevented?

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The answers to these central questions can come only from the most fundamental scientific research, especially in neurobiology. If we fail to find such answers, our descendants will face a plague that produces social and medical burdens of a size that adversely affects their whole way of living. The urgency and scope of the problem means that fundamental neuroscience now shares a responsibility to our future public health comparable to that shouldered by microbiology a half century ago, before vaccines and antimicrobial drugs halted the epidemic and endemic ravages of most infectious diseases. □

Support for unification from atomic physics?

from P. E. G. Baird

UNIFICATION of the weak and electromagnetic interactions in terms of the popular Weinberg-Salam model (*Phys. Rev. Lett.* **19**, 1264; 1967) has received support recently from an atomic physics experiment being carried out at Berkeley (Conti *et al. Phys. Rev. Lett.* **42**, 343; 1979). So far almost all the evidence in favour of this model has come from high energy physics. In 1973, the neutral weak current (n.w.c.) interaction, predicted by unified theories, was detected for the first time in electron-neutrino scattering experiments; the results together with those of subsequent experiments have suggested that this form of the weak interaction (like the charged current interaction responsible for β -decay) is parity non-conserving (PNC), that is it can distinguish left from right. Within the past year clear evidence for PNC has come from a group working at Stanford, who measured an asymmetry of the expected magnitude in the scattering of polarised electrons (at about 20 GeV) from a deuterium target (Prescott *et al. Phys. Lett.* **77B**, 347; 1978). Concurrently with these investigations atomic physicists have been searching for the PNC effects associated with the n.w.c. interaction in atoms. Although many of the experiments have achieved the sensitivity originally estimated for the observation of PNC, the Berkeley experiment is only the second to give a positive result.

The basic idea in the atomic physics investigations is to look for an optical left-right asymmetry. In the absence of a PNC component in the atomic

potential, the pure electromagnetic interaction leads to atomic states of well defined parity. However, the n.w.c. interaction between electrons and nucleus causes some mixing of opposite parity states (particularly $s_{1/2}$ and $p_{1/2}$ states). This alters the character of the radiation arising from atomic transitions and yields a difference in the transition probability for right-handed and left-handed circularly polarised light.

Experimentally, it is one or other of the linked phenomena, optical rotation or circular dichroism, that is being sought.

The size of the PNC effect depends on the strength and type of transition being investigated, the atomic density and the nuclear charge number, Z . The last factor, the Z -dependence, was pointed out in 1974 by the Bouchiat's; they showed that n.w.c. interaction effects in atoms scaled quite rapidly with Z and that an enhancement of up to 10^6 could be gained by studying heavier rather than lighter elements. It is for this reason (in spite of difficulties with atomic calculations) that most atomic experiments are on elements like caesium ($Z=55$), thallium ($Z=81$), lead ($Z=82$) and bismuth ($Z=83$).

For practical reasons the optical rotation experiments are best performed on weak magnetic dipole (M_1) transitions which occur between states of the same parity. The admixture of opposite parity states produces an additional small component of a normally strong (about 10^4 times more intense than M_1) electric dipole (E_1) transition. The magnitude of the M_1 - E_1 interference term which gives rise to optical asymmetry is then comparatively large. In the case of bismuth which is being studied by at least three groups (Oxford, Seattle and Novosibirsk) the expected rotation is about 10^{-7} radians with an atomic density such that 1% of the radiation is absorbed. However, both the Oxford and Seattle groups have failed to detect the expected PNC rotation while the Novosibirsk group, working on the same transition as in Oxford, reported last year findings in broad agreement with the Weinberg-Salam model. Such a discrepancy is unsatisfactory, and more experimental work is needed to resolve it.

Forbidden M_1 transitions are normally selected for the dichroism experiments such as that at Berkeley. Here because the transitions are so weak (10^{-12} of the E_1 rate) the PNC effect can be of order one part in 10^4 .

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In the first of this type of experiment Bouchiat and Pottier (*Phys. Lett.* **62B**, 327; 1976) found that such a transition was too weak to observe directly; they therefore applied a static electric field, E_0 . This does not of itself produce circular dichroism but does mix states of opposite parity and thereby induces an E_1 component which increases the transition rate as E_0^2 . Any PNC induced component now interferes, not with the weak M_1 decay, but with the electric field induced E_1 component. The fractional size of the effect detected is decreased as $1/E_0$ but an acceptable signal (optimised by the field strength) is now obtainable. The Berkeley group has successfully applied this technique to the $6p_{1/2} \rightarrow 7p_{1/2}$ transition of thallium. The dichroism fraction is measured by alternating the sense of polarisation of the input laser beam which is tuned to the transition and then looking for a corresponding change in the upper state polarisation. The transition is in the near ultraviolet (293 nm) necessitating the use of a frequency doubled dye laser. To detect the polarisation change in the upper state a second laser acts as a probe; the polarisation of this is also sequentially changed from right to left. This is claimed to measure any PNC polarisation with 70% efficiency which is about a factor of 10 better than observing the polarisation of the fluorescent radiation. Calibration is an important step in any of these experiments; here it is obtained from the asymmetry data for a given electric field by using the known M_1 rate, but unfortunately no details are given in the paper by Conti *et al.*

The group has studied two hyperfine components: $F=O \rightarrow F'=O$ and $F=O \rightarrow F'=1$. For the former no PNC is expected and indeed the results are statistically consistent with no circular dichroism. The $\Delta F=1$ component, however, is expected to show PNC and the result obtained after some minor corrections is just over twice the standard error from zero. This is consistent with the prediction based on the Weinberg-Salam model and the atomic calculations of Neuffer and Commins (*Phys. Rev. A* **16**, 844; 1977) and Sushkov *et al.* (*JETP Lett.* **24**, 502; 1976) for the size of the PNC induced E_1 component. Of course, higher precision is desirable before claiming unambiguously that PNC in thallium has been observed but the preliminary work shows that the experiment has adequate sensitivity and incorporates many cross-checks against systematic error. If upheld, this result, together with that from Novosibirsk, constitutes the first positive atomic physics evidence supporting the most popular model for the unification of weak and electromagnetic forces. □

Flowering forests

from Michael Kavanagh

THE lowland forests of South-east Asia are the tallest in the tropical world. In peninsular Malaysia about 80% of the emergents that protrude through the dense canopy, sometimes to heights of 200 feet or more, are members of the family Dipterocarpaceae. It has long been known that in these relatively aseasonal forests, the dipterocarps fail to flower most years. Instead, they give rise to profusions of blooms gregariously, at intervals of 3 to 10 years: 'big bang' flowering to tropical forest botanists.

At these times, about 80% of the dipterocarp trees flower, with each tree producing up to four million flowers. The blossoming is highly synchronised. According to H. T. Chan, speaking at the Fifth International Symposium of Tropical Ecology*, populations of each species flower only over a period of 2 to 3 weeks. Among closely-related species, flowering is staggered, although there is some overlapping in time, but mature fruit fall is concurrent. Chan found that the major cause of flower mortality is defective pollination. He also found that all but one of the dipterocarps that he studied are self-incompatible and that trees which stand in clumps produce much more fruit per individual than those which stand alone. He suggested that these clumps are the main breeding groups and that they result from the limited dispersal of winged fruit that is typical of the family. (It seemed to me that this also offered a functional explanation of dipterocarps' limited fruit dispersal.)

Chan's research was conducted at Pasoh Forest Reserve, about 70 km south-east of Kuala Lumpur, as part of a collaborative research and training programme into the reproductive biology of rainforest tree species by members of the Universities of Malaya and Aberdeen. Other members of the team reported related findings. According to S. Appanah, the mighty dipterocarps depend for their pollination on thrips, small insects of the order Thysanoptera. The thrips exist in low numbers in the forest when the dipterocarps are not flowering, during which periods they visit a variety of other flowers. When the trees come suddenly into bloom, the thrips population explodes. It is permitted to do this by the thrips' high fecundity and short life cycle and the fact that the larvae feed on the same flowers that the adults pollinate.

*Held at Kuala Lumpur, Malaysia on 16-21 April 1979.

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It follows that the first dipterocarp to flower, *Shorea macroptera* in this study, faces the problem of a shortage of pollinators. In her presentation, A. Kaur showed that it may overcome this in a remarkable way: normal ova, if not fertilised, may give rise to seedlings through an asexual process known as apomixis. Although Kaur found that several dipterocarp species could produce embryos apomictically, she found that *S. macroptera* was the only one to do so in populations of six species at Pasoh that flowered one after the other. (Kaur *et al.* published a preliminary report last year—*Nature* **271**, 440; 1978.)

The existence of large, complex organisms that are normally sexually reproducing but which can effectively clone themselves from their reproductive parts, should be of interest both to the applied biologists (such as foresters) and to theoreticians. It occurred to me that it added a new dimension to the equation between the benefits of relatedness and the costs of inbreeding depression. In solving the problem of how to reproduce themselves when there are not many thrips around, the trees have hit upon a solution of considerable biological significance. □

A gravitational lens

from F. G. Smith

THE twin quasar reported by Walsh, Carswell and Weymann (this issue of *Nature*, page 381) is the first known example of the gravitational lens effect. This has long been predicted if a massive galaxy should happen to be almost on the line of sight between us and a quasar. The twin, consisting of two very similar objects only 6 arc s apart, was first discovered as a possible optical identification of one of the catalogue of quasars discovered some years ago at Jodrell Bank. The best position of the radio object was found in collaborative work at the US National Radio Astronomy Observatory, but although the coincidence in position was good, Walsh could make no further progress because of the shortage of time on large optical telescopes in the Northern Hemisphere. Eventually he worked in collaboration with Carswell (Institute of Astronomy, Cambridge) at Kitt Peak National Observatory and with Weymann at Steward Observatory. They found almost identical spectra for both objects, and were astonished to find

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that they had identical red shifts, both in emission and in absorption lines. The twins were so identical that they could only reasonably be accounted for by twin images of the same object, exactly as would be expected if a massive galaxy lay on the line of sight.

It happened that this time the new Multiple Mirror Telescope was undergoing trials, including a suitable spectrograph, and fortunately time was available to obtain better spectra of the twins. The results of the observations by Carleton, Chaffee, Davis and Weymann are reported in a footnote to the report and they completely confirm the identity of the spectra. Their beautiful pair of spectra made good material for the symposium on 9 May celebrating the opening of the MMT.

The present observations show only that both twins are point-objects, but higher angular resolution may conceivably show some elongation which would elucidate the actual geometry. If the quasar fluctuates in intensity it will also be interesting to watch for a delay between fluctuations in the two twins.

The discovery of the gravitational lens comes only a few months after the excitement of the orbital changes in the binary pulsar, which demonstrated the existence of gravitational waves. Einstein has indeed been well celebrated in his centenary year. □

Skin toxicology

from Alastair Hay

DERMATITIS accounted for 65% of all industrial injury payments in the UK in 1976 (DHSS Statistics, 1976), and one estimate (Oakley *Health and Safety at Work* 1(8), 48; 1979) puts the number of working days lost because of this ailment at 600,000. The situation is not much better in the United States. A recent report by the Standards Advisory Committee on Cutaneous Hazards Findings (*Job Safety and Health Report* 9, 4; 1979) concludes that 45% of occupational illnesses in the US are due to skin problems, and concedes that this figure is probably too low in view of the recognised under-reporting of these cases. Small wonder, therefore, that this committee recommended a course of actions to the Occupational Safety and Hygiene Administration designed to draw attention to the incidence of skin ailments.

Although the pharmaceutical industry has been interested in skin permeability for a long time, cutaneous toxicology as it relates to the rest of industry is under-researched. There is, however, growing awareness that skin is sensitive to chemical injury and is not an absolute barrier to the absorption of any chemical.

At a recent meeting* M. C. Middleton (Central Toxicology Laboratory, Imperial Chemical Industries Limited) briefly reviewed some of the deficiencies of current test systems for assessing skin toxicity and ways in which they might be overcome.

Current tests for assessing primary irritation are relatively crude. Most are based on the early work of J. H. Draize *et al.* (*J. Pharmac. exp. Ther.* 82, 377; 1944) in which the inflammatory response of skin to chemicals is assessed by their ability to induce redness and oedema. Testing by this method has distinct limitations: assessment is subjective and questionably accurate; opaque and coloured material cannot be visually assessed; it is based on the assumption—never demonstrated—that the extent of the inflammatory response is a measure of all mechanisms of cutaneous toxicity; and the information is of low quality as it says nothing about the mechanism of toxicity.

A more direct approach to the measurement of some of the common manifestations of toxicity in skin is therefore required. Middleton, Dugard and Oliver at CTL suggest that one could measure, for example, the effects of chemicals on the barrier function properties of the stratum corneum, their effect on the normal differentiation pattern of epidermis or that on cell turnover or cellular viability. They are currently evaluating all these suggestions using a combination of biochemical, biophysical and histological techniques.

One aim is to analyse the mechanisms of toxicity. Because the stratum corneum, the final product of epidermal differentiation, is responsible for the 'diffusion barrier' function of skin, an alteration in any aspect of the biology of the epidermis is likely to lead to changes in skin permeability. The sensitive and reproducible techniques for measuring the movement of water or ions across skin could thus be used as general indicators of alterations in epidermal biology (Dugard & Scheuplein *J. Invest. Dermatol.* 60, 263; 1973).

Chemical effects on epidermal differentiation are probably best assessed by looking for disturbances in the normal pattern. These could be

changes in the amount of histidine rich protein, the presence of which appears necessary for the production of normal stratum corneum (Balmain *et al. Devl. Biol.* 60, 442; 1977) or changes in the polyacrylamide gel electrophoresis pattern of keratin—the major end product of terminal differentiation in skin.

Effects on cell turnover may be assessed by thymidine incorporation into DNA and increases are observed following application to the skin of hexadecane (Peters & White *Brit. J. Dermatol.* 98, 30; 1978) and skin irritants such as dibutyltin (Middleton & Pratt *J. Invest. Dermatol.* 71, 305; 1978). However, a more sensitive marker may be those enzymes involved in polyamine biosynthesis (for example, ornithine and S-adenosylmethionine decarboxylases) as they increase in cells undergoing rapid turnover (Jänne *et al. Biochim. biophys. Acta* 473, 241; 1978).

A technique used extensively in clinical biochemistry to assess organ damage was chosen by Middleton to assess frank skin toxicity following chemical insult. Enzyme leakage from cells is a sensitive indicator of cellular viability (Schmidt & Schmidt *Enzymol. Biol. Clin.* 3, 1; 1963) and increases in serum enzyme activity can often identify the site and severity of organ damage.

Enzyme leakage from epidermal cells in response to chemical injury can be detected *in vivo* by the 'blister technique' in which the increase in particular enzymes present in suction induced blister fluid on rat skin after exposure to an irritant chemical is measured.

The blister technique is, however, time consuming and tedious. An *in vitro* epidermal organ culture system has been developed to replace it. Cultures are viable and stable for up to 72 h. Enzyme leakage into the culture medium is used to measure skin damage. Skin dosed with the irritant tributyltin before excision registers a dose-related increase in the leakage of malate dehydrogenase (MDH). MDH and LDH behave similarly both *in vitro* and *in vivo*, but the changes observed *in vitro* can be detected at 1/10 of those occurring *in vivo*. A dose-dependent reduction in glucose utilisation, oxygen consumption and ATP levels also occurred with tributyltin, indicating a reduction in cellular viability. Thus, says Middleton, enzyme leakage in his *in vitro* system may offer a simple, sensitive and early indicator of changes in cell viability of skin exposed to cutaneous toxins, changes which so far correlate with those seen *in vivo*.

Middleton and his colleagues at CTL believe that the approaches outlined

*Organised by the British Toxicology Society and held at the University of Warwick on 5 April 1979.

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above could, if successful, ultimately lead to the development of test systems which would produce better quality information on the cutaneous toxicity of irritant chemicals and would help target any in depth mechanistic research if so desired.

It is not only skin toxicologists who want a more realistic approach to the assessment of toxicity. Many tests currently advocated by government regulatory authorities are a blanket approach to the problem yielding information, in many cases, of little value. They argue that a more selective approach is required with the emphasis on the quality of the information rather than quantity. □

Eukaryotic promoters?

from N. J. Proudfoot

By analogy with bacterial genes, eukaryotic genes should have a well-defined promoter region somewhere in front of the start of the mRNA sequences at which transcription is initiated and which is crucial in the regulation of gene expression. But eukaryotic promoters are proving elusive.

The development of sophisticated recombinant DNA techniques has allowed the isolation of a rash of animal genes, albeit rather atypical in function. The genes for globin, immunoglobulin, ovalbumin, histone and several animal viruses, to mention but a few, have all been integrated into lambda or plasmid derivatives and gone into mass production. The milligram quantities of pure animal gene obtained in this way are now under intense scientific investigation, both of their structure and more problematically their function. One clear aim is to define the beginning and end of each gene in the hope of discovering common control features. Several groups have therefore carried out extensive sequence analysis of the 'ends' of genes as defined by the mRNA or protein sequence, greatly assisted by the new DNA sequencing procedures from both sides of the Atlantic.

A few years ago it looked very much as though the transcription initiation point or promoter was in general a very long way away from the start (the 'cap') of the final messenger RNA (mRNA) or the initiating AUG of the protein coding sequence. High molecular weight heterogeneous nuclear

RNA was at least in some cases proved to be the precursor to cytoplasmic mRNA, so that the disparity in size of these two RNA classes seemed to require a promoter extremely distant to the gene in question. The recent discovery of gene inserts (intervening sequences or introns) put an end to such models. It has become abundantly apparent since, that the initial or primary transcript of a gene is longer than the mRNA, largely because all the inserts (as many as seven in the case of chicken ovalbumin) are transcribed and then subsequently chopped out (spliced). The promoter sequence of an animal gene is therefore likely to be close to the beginning of the mRNA sequence. In a recent issue of *Cell* two papers describe sequence homologies between possible promoter regions of the RNA polymerase II and III genes (Konkel *et al.* *Cell* 15, 1125; 1978; Korn & Brown *Cell* 15, 1145; 1978). Five polymerase II genes share a partial homology of 12 nucleotides before and including the 'cap site' of each mRNA. It is, however, difficult to decide whether or not the homologies are good enough to warrant the title of promoter. Perhaps 'capping site' is a better term as favoured by the authors. The polymerase III genes, mainly different species of 5S RNA, are perhaps more strikingly homologous with boxes of complete homology between several of the genes at least.

In contrast, many prokaryotic promoters have been sequenced and perhaps the most impressive homology found is the so-called 'Pribnow box'—TATPuATG. David Hogness of Stanford has pinpointed a rather similar sequence homology recently in putative promoter regions of eukaryotic histone genes. His laboratory's analysis of *Drosophila* histone genes has revealed that in all probability each of the six genes which compose a single repeating unit (repeated about 100 times) has a separate promoter. He notes that in each case a sequence closely resembling TATAAATA is present about 70 nucleotides from the initiator triplet ATG. Assuming a normal length 5' non-coding region sequence of 50 nucleotides this homology is at a reasonable promoter position. Similarly sea urchin histone genes and a few other non-histone genes—silkworm fibroin, mouse and rabbit α -globin, adenovirus late genes and yeast isocytochrome *c*—each display partial homology with the 'Hogness box' in promoter regions. Finally, Gannon *et al.* (*Nature* 279, 428; 1979) have recently identified the 5'-end of chicken ovalbumin mRNA in the chick genome. This proved difficult as the 5' three-quarters of the 5' non-coding region of this already over-spliced mRNA are separated from the rest of the gene by

1.5 kilobases. Sure enough, 25 nucleotides away from the cap, beyond Konkel *et al.*'s 'capping sequence', is a somewhat deformed 'Hogness box'. From these few eukaryotic promoter sequences, the emergence of a partially homologous AT-rich sequence or box common to all eukaryotic genes does seem compelling. Of course, the essential information lacking in this argument is that RNA polymerase II does indeed interact with this sequence during initiation of transcription. These data are available for the prokaryotic 'Pribnow box' and bacterial RNA polymerase.

Another and more direct way to sort out this problem has recently been initiated in several laboratories with rather startling results. If a eukaryotic gene could be expressed *in vitro* then the requirement for gene transcription of this putative promoter sequence can be tested directly. At a recent meeting in Colorado Don Brown of the Carnegie Institution, Baltimore, described elegant experiments involving the expression of *Xenopus* 5S genes in extracts of *Xenopus* oocyte nuclei. He found that 5S RNA was produced even when the highly conserved 'promoter boxes' were removed by chopping off the promoter region of the 5S gene with restriction enzymes. Such a result could be explained away by quantitative arguments. For instance, a very high concentration of 5S gene, even though lacking a promoter, might somehow drive the transcriptional apparatus into a low level of specific initiation. It remains true that there is no absolute requirement for this putative promoter. It seems possible that the days of the Hogness box may also be numbered. □

NATURE

A hundred years ago

THE carrier-pigeon service is now in full operation in France, and has been placed under the direction of the head of aerial communication. The number of birds fed by the Government is 6,000. These pigeons are located in Paris and twelve other large fortified towns. A number of soldiers and officers have been taught the art of pigeon breeding, and carriers are constantly sent from place to place. The Minister of Public Instruction and the Minister of Agriculture have established prizes for pigeon races.

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review article

Recent developments in tunable lasers for spectroscopy

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Advances in tunable lasers over the past few years are reviewed. Particular emphasis is placed on their application to spectroscopy.

THE technology of tunable lasers has advanced quite rapidly in the past few years (see ref. 1). The most successful spectroscopic coverage has been achieved in the visible and near IR region with dye lasers, but tunable coherent radiation is now available from one source or another from the UV at one end of the spectrum to the middle IR. In addition, optical pumping of a variety of different gases has provided a 'line tunable' laser source at a very large number of discrete frequencies throughout the far IR region. Perhaps the outstanding technical problem at present is that of achieving truly continuous tuning for middle and far IR laser radiation.

Spectroscopy, and the understanding that is obtained on a microscopic level from spectroscopic experiments, is enjoying no less a revolution than that initiated by the first fixed frequency lasers. Also, when the power levels of continuous coherent radiation approach 0.1–1 W the new techniques of nonlinear spectroscopy become possible. New techniques, for example for isotope separation and photo-physics and chemistry, are being widely studied.

Here we briefly review current developments in tunable laser systems with emphasis on their application to spectroscopy. A more general review of all the principle systems is given elsewhere². For convenience we divide the subject into IR lasers (excluding dye lasers) and near UV through visible to near IR lasers. We consider the following systems: semiconductor diode, spin-flip, high pressure gas, three- and four-wave mixing, optical parametric oscillator, solid state F centre, excimer and dye lasers. We retain our earlier definition of tunable lasers to mean lasers which are broadly tunable over 100 cm^{-1} or more. Of course, in very high resolution applications the phenomena of mode hopping is observed, that is, the broad tuning range is made up of a large number of small continuous tuning ranges. This is true for all Fabry–Perot laser structures. The lasers fall broadly into two categories; primary lasers (such as pulsed dye, semiconductor, excimer and high pressure molecular gas lasers) which can be incoherently pumped and are tunable over their broad gain bandwidths; and secondary lasers (such as the spin-flip laser and optical parametric oscillator) which must be coherently pumped by a fixed frequency laser, with tuning achieved by a nonlinear process—in this case Raman scattering and frequency difference mixing.

Diode lasers

Perhaps the most compact of all tunable lasers is the semiconductor diode, which is now available commercially in the form of lead salt alloys at selected wavelengths in the range 2.7–25 μm

(refs 3, 4). They can operate very efficiently at low power levels where they have been used extensively for IR molecular absorption spectroscopy. However, it is difficult to scale up the output power from a single diode because of catastrophic degradation of the Fabry–Perot faces, and peak output powers from double heterostructure stripe geometry devices are usually limited to $\ll 1\text{ W}$. These structures, which produce both carrier and electromagnetic wave confinement, are necessary to reduce the threshold power, for example, for operation at liquid nitrogen temperatures, and to achieve good optical mode quality. Typical single mode output powers for c.w. ternary lead salt lasers are in the range 0.1–1 mW. By choosing alloys of different semiconductor compounds it is possible to achieve laser operation centred anywhere between 0.6 and 32 μm . An individual laser can also be tuned by magnetic field, hydrostatic pressure or temperature variation. The problems of fine tuning and ultra-high resolution single mode laser spectroscopy mean that in practice a single diode will only be used to cover a region of a few tens of wavenumbers. Diode lasers have been used for heterodyne spectroscopy, but the single mode powers now available make them only marginal for astronomical heterodyning applications in the 10 μm 'window' region. Jennings⁵ has recently reported diodes used for nonlinear spectroscopy.

A spectacular spectroscopic application has been in the use of lead tin telluride diodes to probe the multi-photon excitation of a Q-switched CO_2 laser in the molecular gas SF_6 . This double resonance study provides the first high resolution observation of the resulting excited state transitions in this molecule (Fig. 1), where linewidths between 10 and 100 MHz are typical.

Spin-flip lasers

The spin-flip laser (SFL) is based on stimulated Raman scattering from conduction electrons in a semiconductor in the presence of an external magnetic field. The elementary excitation of the medium derives from the Zeeman spin splitting of the lowest conduction band Landau level, so that the Stokes radiation has energy equal to the pump laser energy minus $g^*\beta B$, where g^* is the effective g-factor, β is the Bohr magneton and B the magnetic field. In the presence of spin orbit coupling, and a small conduction electron effective mass, g^* can be very large with resulting sizeable tuning range (about $1.6\text{ cm}^{-1}\text{ kG}^{-1}$ for InSb, with g^* of ~ 50). Further, the cross-section may be very large because the Thomson cross-section, σ_T^* , of an electron in a semiconductor exceeds that of a free electron in the ratio $(m/m^*)^2$; if the effect is further enhanced by making the frequency of the exciting photons approach the energy of the

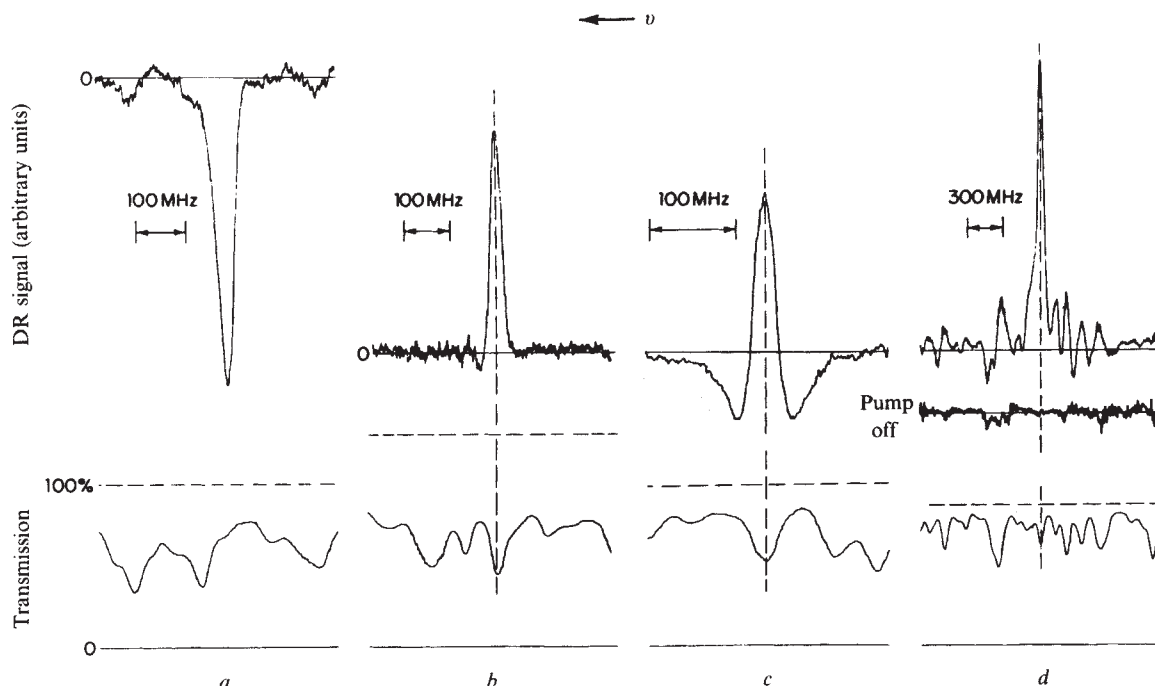


Fig. 1 Lineshapes of four representative double resonance (DR) signals taken with a PbSnTe diode laser probe and CO₂ laser pump lines: a, P(18); b, P(16); c, P(22); and d, P(12). Simultaneous average SF₆ transmission spectra are shown below (after Moulton *et al.*¹⁶).

direct band gap, the cross-section can approach one million times that of the free electron. As the electronic processes are also less frequency-dependent than those of phonons (which vary as ω_p^2) it is possible to obtain stimulated scattering with IR laser pumps where the wavelength is matched to the band gap of several interesting semiconductors. By far the majority of SFL applications to date have been with the technologically well controlled material InSb as the active medium. Apart from the ease of obtaining the cavity material, this has the advantage that the band gap is matched to the CO laser near 5 μm which is used as the pump for c.w. operation (where threshold powers as low as a few milliwatts have been achieved). Coarse tuning has been achieved c.w. from 5.2 to 6 μm with about 1 W Stokes output power, and pulsed (using a CO₂ laser pump) from 9 to 14.6 μm from first and second Stokes and anti-Stokes output; the maximum Stokes peak power is about 1 kW. It has not been possible to achieve c.w. output from the InSb SFL in the 10 μm region, but very low power low field c.w. output has been reported from HgCdTe matched to the 9.6 μm CO₂ laser pump⁶.

As with the diode laser, the SFL has by now been widely used for linear spectroscopy (see ref. 8), and recently in excited state

double resonance studies of molecules⁹. Also, the somewhat greater output power available in the 5 μm region has been used for nonlinear, Lamb dip, spectroscopy¹⁰, and photochemistry of iron carbonyl compounds, as shown in Fig. 2.

Other IR lasers and parametric oscillators

High pressure IR molecular gas lasers have a continuous gain profile due to the overlap of pressure-broadened adjacent rotational lines. Insertion in the laser cavity of a suitable tuning element then results in a tunable laser. C.w. operation has not yet been achieved, probably because of fast collisional deactivation times. Various means of excitation have been used. Electron beam excitation of CO₂ at about 15 atmospheres produces scalable output energies of ~ 0.1 J in the wavelength region 9–12.3 μm or alternatively pumping can be achieved in two steps by UV preionisation plus a sustainer voltage pulse giving output energies of several Joules per litre. Optically pumped N₂O and CO₂ lasers at pressures up to 42 atmospheres have been produced by using an HBr TEA laser pump¹¹. An alternative scheme of Kildal and Deutsch¹² utilises a frequency-doubled CO₂ TEA laser to excite the $\nu = 1$ vibrational mode of CO which can then produce laser action in other gases by collisional transfer of vibrational energy to other molecular levels within an energy of $\sim kT$ of the $\nu = 1$ CO mode.

Three-wave frequency mixing has been used to generate tunable sum and difference frequency radiation from the UV to the far IR in a variety of different nonlinear crystals, but very few of these schemes have been utilised for high resolution spectroscopy. One exception to this has been the work of Pine¹³ who obtained c.w. laser spectroscopy from 2.2 to 4.2 μm by difference-mixing an argon ion laser with a c.w. dye laser in LiNbO₃. Output powers of ~ 1 μW were achieved with spectral resolution of better than 10 MHz (Fig. 3). Unfortunately, most crystals with low optical losses in the visible have poor IR transmission. Exceptions to this are CdSe and the ternary compounds AgGaS₂, AgGaSe₂ and CdGeAs₂. By mixing pulsed dye lasers or optical parametric oscillators (see below), it is possible to get to about 25 μm in this way, with spectral resolution typically of a few tenths of wavenumber. The major application of this type of device is probably to photochemistry, rather than to high resolution spectroscopy. Four-wave mixing processes have also been demonstrated in a large variety of

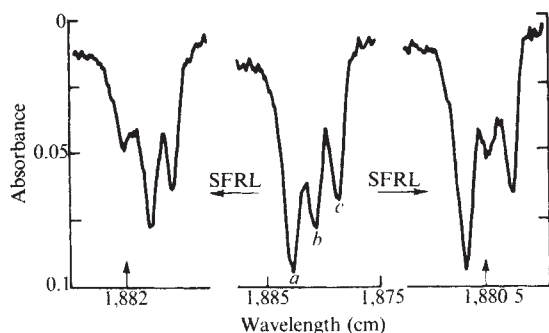


Fig. 2 Absorption spectra of matrix isolated Fe(CO)₄. Non-degenerate vibrational modes give rise to several different bands in the matrix. Tuning the spin-flip Raman laser (SFRL) to band a reduces band a, leaving b and c unchanged. Similarly tuning to band b reduces band b, leaving a and c unchanged. This indicates that site-selective photochemistry is occurring (after Poliakov *et al.*³⁴).

solids, liquids and metal vapour gases, with tunable laser pumps, as has multiple order Raman scattering in high pressure gases (see ref. 1 for a recent bibliography). As these depend on third order optical nonlinearity, in general high pumping intensities are required and application is confined to pulsed operation.

The second order nonlinear polarisability that describes the sum and difference three-wave frequency mixing is also responsible for the parametric oscillator output. The pump laser frequency ω_p generates waves of two other frequencies in a nonlinear anisotropic crystal, ω_s and ω_i , known as the signal and the idler. These are subject to the energy conservation condition, $\omega_p = \omega_s + \omega_i$, and the wavevector phasematching condition for coherent interaction, $\mathbf{k}_p = \mathbf{k}_s + \mathbf{k}_i$. This means that for collinear propagation, $\omega_p n_p = \omega_s n_s + (\omega_p - \omega_s) n_i$, where n is the frequency-dependent refractive index, so that tuning can be accomplished by using the angular or temperature dependence of the birefringence of the anisotropic crystal. One can operate the oscillator in two distinct ways depending on whether the mirrors are reflecting at both signal and idler wavelengths (doubly resonant oscillator or DRO) or reflecting at only one wavelength (singly resonant oscillator or SRO). The DRO has a lower threshold pump power than the SRO and has been made to operate c.w. in limited wavelength ranges shorter than 2 μm . It suffers, however, from the disadvantage that instability occurs as a result of the need to match the cavity frequencies for both the signal and idler waves. This problem does not exist for the SRO, which so far has only been operated pulsed. Recent advantages in crystal growth technique have made it possible to operate a LiNbO_3 SRO pumped directly with a Nd:YAG laser with near 500 mJ of a single mode pulsed output energy, resulting in tunable outputs of up to 100 mJ (refs 7, 14). With this device, involving computer controlled operation of the SRO together with an intra-cavity grating and etalon, it is possible to obtain tunable output from about 1.5 to 4.0 μm , with linewidth of a few tenths of a wavenumber. Various means have been used to extend this further into the IR, including difference mixing in CdSe and frequency shifting by stimulated Raman scattering in gaseous hydrogen. Again the application appears most promising for moderate-resolution photochemistry rather than ultra-high resolution spectroscopy.

Finally, several alternative solid state lasers have shown some promise as IR sources. For example, Mollenauer and Olson¹⁵ have demonstrated c.w. laser action from colour centres in the alkali halides, where luminescence bands cover the region 1.4–3.4 μm . Two c.w. tunable lasers were obtained by pumping $F_A(\text{II})$ centres in $\text{KCl}:\text{Li}$ and $\text{RbCl}:\text{Li}$ with a krypton ion laser at 6,471 Å. Typically tuning was from 2.5 to 2.9 μm at operating temperatures between 77 K and 200 K, providing an IR solid state analogue of the c.w. dye laser. Another system has been transition metal impurities in such crystals as MgF_2 . Recently Moulton *et al.*¹⁶ have improved the $\text{Ni}:\text{MgF}_2$ laser by using a c.w. Nd:YAG laser as the excitation source in a geometry similar to the F-centre laser. Nearly 2 W of continuous wave power was

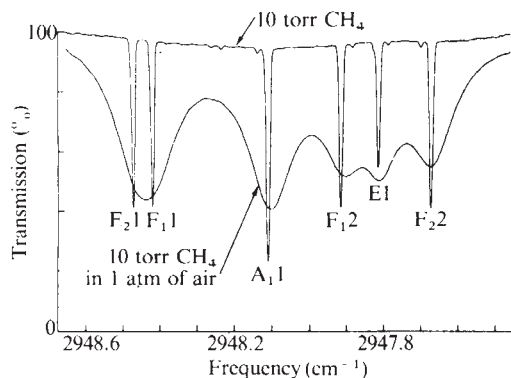


Fig. 3 Absorption spectra of methane in the Doppler limited and pressure broadened regions taken with a LiNbO_3 difference frequency laser spectrometer (after Pine¹³).

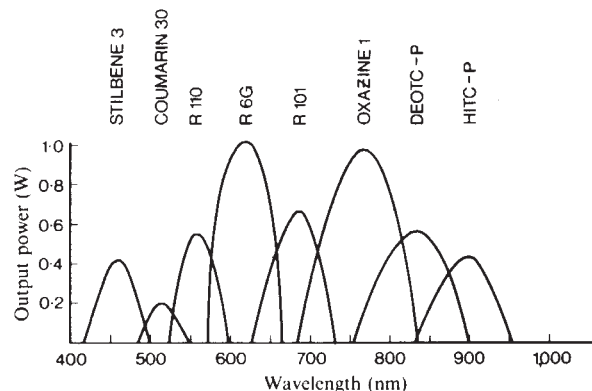


Fig. 4 Approximate tuning ranges and output powers of a selection of c.w. laser dyes covering the spectrum from 410 to 1,000 nm. (The following pump powers have been assumed: 4 W all line krypton for 1 R dyes, 4 W all line argon for rhodamine dyes and 2 W UV for the blue dyes).

obtained, tunable between 1.60 and 1.74 μm . The $\text{Ni}:\text{MgF}_2$ device is now available commercially and it is possible that with the further potential of efficient pumping from a GaAs diode laser and appropriate nonlinear frequency shifting it may lead to a more flexible and higher power tunable IR source than available from the lead salt diodes.

Dye lasers

In the visible region of the spectrum dye lasers still represent the most widely used tunable sources. Since 1975² there have been several significant developments which have improved the performance of, in particular, the c.w. dye lasers. First, there has been the availability of efficient laser dyes operating in the near IR down to 950 μm . The polymethine dyes DEOTC and H1TC¹⁷ and their perchlorate modifications DEOTC-P and H1TC-P besides extending the available tuning range have high gain and efficiency and are reasonably stable with operating lifetimes of several days. These laser dyes require longer wavelength pumping and, as with the colour centre lasers, a krypton ion laser with optics to ensure multiline operation in the red is the most effective source. At the other end of the spectrum the new dyes based on stilbene provide significant advantages over the coumarins¹⁸. In particular stilbene 3 is more stable, has a higher gain and is more efficient but most importantly provides a much greater spectral coverage than any single coumarin derivative. Figure 4 illustrates the performance typical of several dyes including stilbene 3 and the IR dyes.

One of the main difficulties in obtaining high power single frequency operation from the astigmatically compensated three mirror cavity configuration normally used in c.w. dye lasers is the phenomenon of spatial hole burning. This occurs as the result of the standing wave laser field in the dye stream producing regions of low gain (high field) spatially separated from regions of high gain (low field). The latter regions tend to encourage oscillation on axial modes sufficiently separated in frequency from the initial mode to ensure that their standing wave patterns overlap the high gain regions. This situation is inherently unstable and requires strong mode selecting elements inside the cavity to overcome it. It was realised some time ago that unidirectional travelling wave operation in a ring cavity configuration would overcome this problem¹⁹ though it is only within the last year that ring c.w. dye lasers have been commercially available. Around 100 mW of single mode power can be readily obtained using simple intra-cavity dispersion (coarse tuning with a birefringent filter and a single etalon). Part of the difficulty in introducing these systems has been the development of a low loss unidirectional device to force single travelling wave operation around the ring. The construction of such a device, based on Faraday rotation, has been described in detail elsewhere²⁰.

Further extension of the tuning range of c.w. dye lasers can be accomplished by intra-cavity harmonic generation and reasonable powers (~ 10 mW) have now been demonstrated with a limited tuning capability around 300 nm (ref. 21). The development of the higher power ring laser systems together with the improvements in blue dyes should encourage the extension of nonlinear techniques such as harmonic generation and difference mixing (as mentioned earlier) to provide modest powers between 4,000 nm and 250 nm.

Ultra-high resolution spectroscopy with c.w. dye lasers has improved by at least an order of magnitude since 1974. To achieve linewidths around 100 kHz requires extensive ancillary equipment as is clear from the system operated by Gerhardt and Timmerman²². The output from their dye laser was first locked to a stable external reference cavity. To achieve tuning this cavity was in turn locked to a He-He laser itself frequency offset-locked to a Lamb dip stabilised He-He laser. The authors demonstrated a frequency calibration accuracy of ± 50 kHz in saturation spectroscopy measurements of the hyperfine splitting of sodium. Other atomic systems besides the popular sodium are now being examined and a simpler form of heterodyne spectroscopy, applicable in some cases, has been used to measure the even-even isotope shifts in samarium²³. In these experiments two dye lasers were used, each locked to the resonance scattering maximum of two isotopes present in a collimated atomic beam. The two laser beams were heterodyned and the beat frequency, giving the isotope shift, was measured to ± 200 kHz, an accuracy some two orders of magnitude better than previous conventional methods had given, and sufficient to detect discrepancies between observed and predicted results. This and earlier work on isotope shifts in krypton²⁴ obtained by polarisation spectroscopy and shown in Fig. 5, show the present capabilities of high resolution c.w. dye lasers.

Mode-locking of c.w. dye lasers to produce a continuous train of ultrashort pulses ($\sim 10^{-11}$ – 10^{-12} s duration) has had an impetus through the introduction of synchronous pumping. This concept is based on mode-locking the pump ion laser to give pulses of around 200 ps duration. By arranging for the dye laser cavity to have the same optical length as the pump laser cavity, the establishment of a similar train of pulses from the dye laser is encouraged²⁵. It is, however, possible to obtain pulses from the dye laser of considerably shorter duration than the 200 ps ion laser pulses as the leading edge of the dye pulse can saturate the gain available in the active stream. Although this approach requires the careful control of the two cavity lengths it eliminates the need for passive mode-locking²⁶ with its associated difficulties of another dye stream, focusing optics and the matching of suitable saturable absorbers with each lasing dye. Utilising electro-optic cavity dumping to switch out selected pulses can provide output powers up to 1.5 kW at repetition frequencies ~ 1 MHz. This form of mode-locking could in principle operate with any of the established c.w. laser dyes²⁷.

No particularly significant advances seem to have been made in the area of pulsed dye lasers though both flashlamp pumped and N₂ laser pumped commercial devices have seen improvements in their specifications particularly in repetition frequency, and ease of wavelength control and dye interchange. Average output powers have increased with specialised flashlamp and cell design to >100 W (ref. 28), an aspect of tunable lasers which may prove important in their application to large scale isotope separation. Direct electrical excitation of vapour phase dye lasers has yet to be achieved. However, recent electron beam pumping measurements by Marowsky *et al.*²⁹ are a further step in this direction.

UV sources

In the UV region harmonic generation from pulsed visible dye lasers still provide the most readily available source and the greatest tuning flexibility. The excimer and exciplex lasers (see ref. 30) offer alternative sources which extend further into the vacuum UV region below 200 nm, and provide not only high power with which to pump shorter wavelength dyes but also a

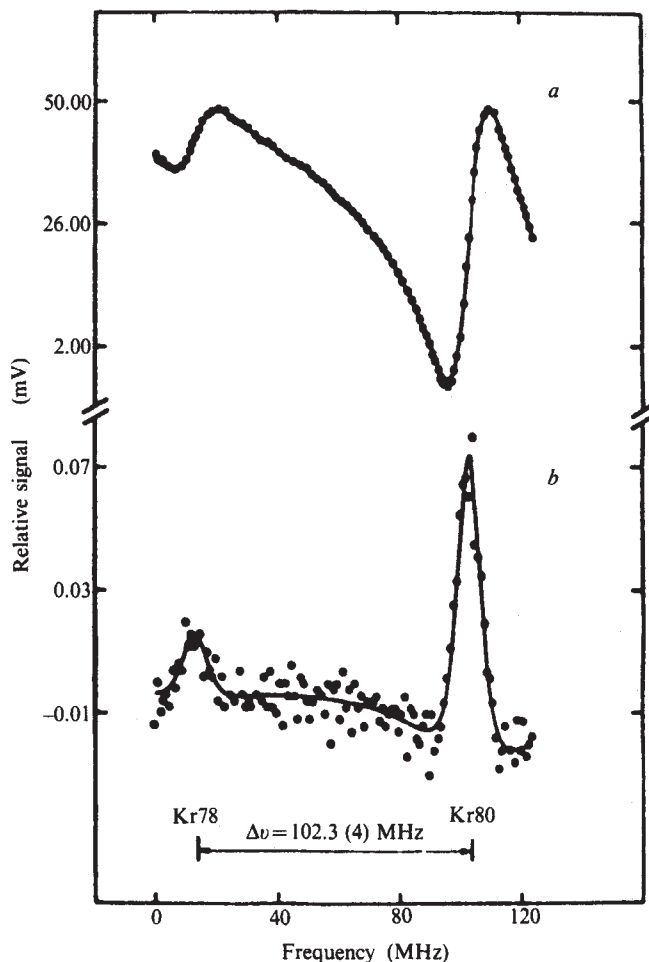


Fig. 5 Polarisation spectrum (a) and its derivative (b) of two krypton isotopes. The isotope shift is shown explicitly (after Gerhardt *et al.*²⁴).

limited direct tuning capability. These systems which are sometimes all known as excimer lasers depend on the formation of an excited state molecule from either two rare gas atoms (excimer, for example, Xe-Xe) or a rare gas-halogen atom combination (exciplex, for example, Kr-F). In both cases the important feature is that the ground state 'molecule' is unstable and rapidly dissociates ensuring a near zero lower laser level population. Both electron beam pumping and transverse electric excitation have been used as pumping methods to provide the necessary high energy short duration excitation pulse. Much emphasis has been placed on obtaining high output powers and extending the range of atomic combinations which form exciplexes and less on developing their tuning capabilities, which for xenon is over a band $\sim 3,000$ cm⁻¹ wide, centred at 172 nm. The power available from these lasers and harmonic generation in atomic vapours promises to provide tunable radiation around several discrete wavelengths in the 10–100 nm region³¹.

Free electron laser

Finally, we should mention the free electron laser which has been demonstrated at Stanford³² and more recently the Naval Research Laboratories³³. Although limited to establishments with the necessary electron accelerator facility such a laser is potentially tunable throughout the visible and IR with a high output power capability. We note, however, that the regions of the spectrum not covered by tunable lasers as mentioned in our previous review² have now been reduced significantly through steady advances in less exotic but more practical ways.

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articles

0957 + 561 A, B: twin quasistellar objects or gravitational lens?

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0957 + 561 A, B are two QSOs of mag 17 with 5.7 arc s separation at redshift 1.405. Their spectra leave little doubt that they are associated. Difficulties arise in describing them as two distinct objects and the possibility that they are two images of the same object formed by a gravitational lens is discussed.

SPECTROSCOPIC observations have been in progress for several years on QSO candidates using a survey of radio sources made at 966 MHz with the MkIA telescope at Jodrell Bank. Many of the identifications have been published by Cohen *et al.*¹ with interferometric positions accurate to ~2 arc s and a further list has been prepared by Porcas *et al.*² The latter list consists of sources that were either too extended or too confused for accurate interferometric positions to be measured, and these were observed with the pencil-beam of the 300 ft telescope at NRAO, Green Bank at λ 6 cm and λ 11 cm. This gave positions with typical accuracy 5–10 arcs and the identifications are estimated as ~80% reliable.

The list of Porcas *et al.* includes the source 0957 + 561 which has within its field a close pair of blue stellar objects, separated by ~6 arc s, which are suggested as candidate identifications. Their positions and red and blue magnitudes, m_R and m_B , estimated from the Palomar Observatory Sky Survey (POSS) are given in Table 1 and a finding chart is given in Fig. 1. Since the images on the POSS overlap, the magnitude estimates may

be of lower accuracy than normal, but they are very nearly equal and object A is definitely bluer than object B. The mean position of the two objects is 17 arc s from the radio position, so the identification is necessarily tentative.

Observations

The two objects 0957 + 561 A, B were observed on 29 March 1979 at the 2.1 m telescope of the Kitt Peak National Observatory (KPNO) using the intensified image dissector scanner (IIDS). Sky subtraction was used with circular apertures separated by 99.4 arc s. Some observational parameters are given in Table 2. The spectral range was divided into 1,024 data bins, each bin 3.5 Å wide, and the spectral resolution was 16 Å. After 20-min integration on each object it was clear that both were QSOs with almost identical spectra and redshifts of ~1.40 on the basis of strong emission lines identified as C IV λ 1549 and C III] λ 1909. Further observations were made on 29 March and on subsequent nights as detailed in Table 2. By offsetting to observe empty sky a few arc seconds from one object on both 29 and 30 March it was confirmed that any contamination of the spectrum of one object by light from the other was negligible.

Table 1 Positions and magnitudes of 0957 + 561 A, B

Object	RA	Dec (1950.0)	M_R	M_B
0957 + 561A	09 57 57.3	+56 08 22.9	17.0	16.7
0957 + 561B	09 57 57.4	+56 08 16.9	17.0	17.0

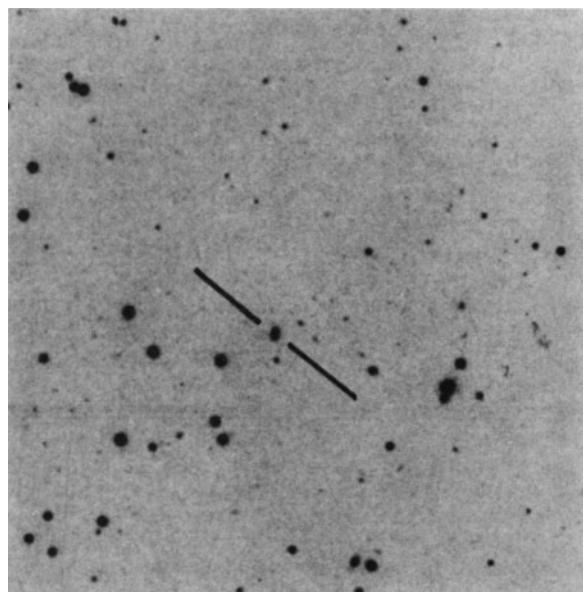


Fig. 1 Finding chart for the QSOs 0957 + 561 A and B. The chart is 8.5 arc min square with the top right hand corner north preceding and is from the E print of the POSS.

On 1 April the spectral range was altered slightly by tilting the grating to cover the anticipated redshifted wavelength of Mg II $\lambda 2798$ which was just beyond the limiting wavelength on previous nights.

The spectra obtained on 1 April are shown in Fig. 2. Data on observed spectral lines are given in Table 3. These were taken from the spectra using the interactive picture processing system (IPPS) which makes a linear interpolation between two selected continuum points and calculates the centroid and equivalent width of the emission above the interpolated line. Data from all three nights were used in compiling Table 3; that on 1 April had double the signal-to-noise ratio of the other two nights and was weighted accordingly. The O IV] $\lambda 1402$ line is outside the spectral range of Fig. 2 but was present in data taken on the other two nights. Although we believe that Mg II $\lambda 2798$ is detected in the data of Fig. 2 for 0957 + 561B, and He II $\lambda 1640$ is also detected taking into account all three nights' data, the low signal-to-noise ratio and poorly defined continuum prevent us deriving useful observed wavelengths or equivalent widths.

The data on the C IV $\lambda 1549$ and C III] $\lambda 1909$ lines are much more accurate than those on the other lines and we believe the r.m.s. errors in the observed wavelengths of the centroids of these lines are not greater than 3 Å while the r.m.s. errors in the equivalent widths are estimated to be 7 Å. Within the limits of observational error, the corresponding lines in each object are identical in observed wavelength and equivalent width. For each object there is a difference in the redshift derived from the C IV and C III] lines which is significantly greater than the combined r.m.s. error in each. This may be associated with the problem of giving a precise meaning to the redshift of a broad line of somewhat irregular shape. The mean values of the redshift from the C IV and C III] emission lines are 1.4054 for A and 1.4047 for B, the difference being within the errors of measurement.

Although no attempt was made to carry out accurate spectrophotometry, some characteristics of the continua seem fairly well defined. Below about 5,300 Å they appear to have identical shapes, with QSO A brighter than B by 0.35 mag. Above 5,300 Å, however, the flux from B rises more steeply than that from A and they are equal at $\sim 6,500$ Å. These results are consistent with the magnitude estimates of Table 1.

The pair of QSOs provides unusual opportunity to investigate the origin of absorption lines in QSO spectra, a matter which is still in dispute. Accordingly, spectra having a resolution of about

Table 2 List of IIDS observations with 2.1 m telescope

Date (1979)	Aperture (arc s)	Seeing (arc s)	Spectral range (Å)	Integration time, each object (min)
29 March	3.4	4	3,200–6,700	40
30 March	1.8	1	3,200–6,700	20
1 April	3.4	3	3,500–7,000	60

2 Å were obtained of both QSOs on 30 March using the image tube spectrograph attached to the University of Arizona 2.3 m telescope. As in the observations described above, the seeing during the observations was sufficiently good for contamination of the spectrum of one QSO by the light from the other to be negligible. A portion of the tracings of the two plates covering the C IV emission line region is shown in Fig. 3. The absorption lines which have been identified are indicated on the figure, and the measured wavelengths (using a Grant measuring engine) are presented in Table 4, with the corresponding redshifts. The wavelengths of the C IV emission lines given in Table 4 were measured from the tracings by smoothing over the noise and finding the centre of symmetry for each line. Comparison with Table 3 shows that the agreement in wavelength for the C IV emission lines between the two sets of observations is within the errors of measurement.

Low ionisation absorption systems (ones with Si II and Al II strengths $> \text{C IV}$ strengths) are clearly present at $z_{\text{abs}} = 1.390$ in both QSOs. Even in the low resolution IIDS spectrum of QSO A there is clear evidence for Fe II $\lambda 2383$ and Mg II $\lambda 2798$ absorption. Fe II $\lambda \lambda 2600$ and 2344 are possibly also present. Weak and possibly real absorption lines also appear in the image tube spectrum at $\lambda 3536.1$ and $\lambda 3835.1$ of QSO A. The features at $\lambda 3835.1$ and $\lambda 3844.0$ have a separation close to that of the Mg II doublet (at redshift 0.372). However, $\lambda 3844$ is already identified with Fe II $\lambda 1608$ in the 1.390 system so that the evidence for Mg II at 0.372 is not convincing. In QSO B, the

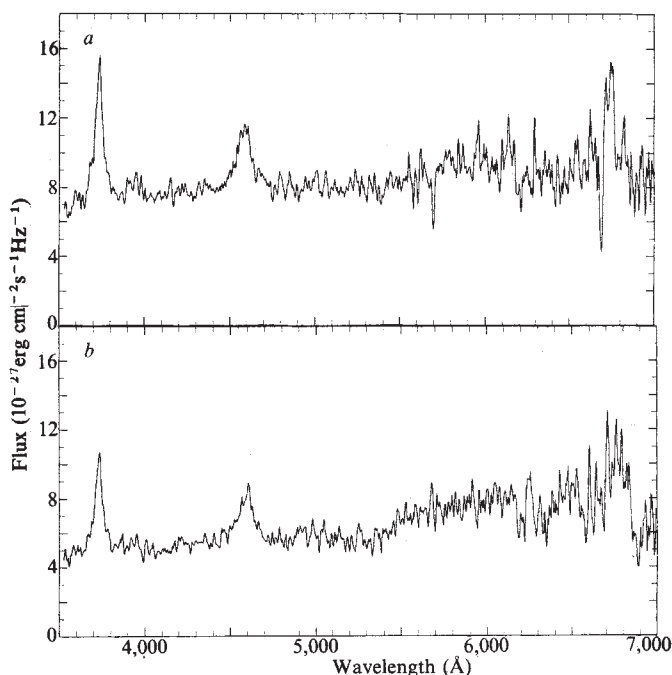


Fig. 2 IIDS scans of 0957 + 561 A (a) and B (b). The data are smoothed over 10 Å and the spectral resolution is 16 Å.

absorption lines seem to be weaker than in QSO A on the basis of both the plate and IIDS data and none are seen in the low resolution spectrum. Unfortunately, a dust speck on the mask used to suppress image tube noise obliterated the Si II line in the spectrum of this object.

The difference between the two absorption redshifts amounts to a velocity difference $\Delta V_{\text{abs}}(B-A)$ of only about $+45 \text{ km s}^{-1}$. However, in addition to the errors in estimating the line centres, somewhat larger errors occur in the zero point of the wavelength scales from plate to plate amounting typically to 100 km s^{-1} . As a result the difference between the absorption line redshifts in QSO A and QSO B cannot be considered significant.

The image tube data on the C IV emission lines give a velocity difference $\Delta V_{\text{em}}(B-A)$ of $+265 \text{ km s}^{-1}$. This is also subject to the zero point error, but the major source of error is the uncertainty of $\sim 1.5 \text{ \AA}$ ($=120 \text{ km s}^{-1}$) in estimating the position of each line centre. The IIDS data on the C IV and C III] lines permit two independent estimates of the velocity difference leading to a mean $\Delta V_{\text{em}}(B-A) = -95 \text{ km s}^{-1}$ with an error slightly larger than for the image tube data. Combining both sets of data, the resulting $\Delta V_{\text{em}}(B-A)$ is $+120 \pm 150 \text{ km s}^{-1}$. Again, the difference between the emission redshifts in the two QSOs cannot be considered significant.

Table 3 Wavelengths, equivalent widths (EW) and derived redshifts from IIDS 2.1 m observations

	λ_{em}	O IV]	C IV	He II	C III]	Mg II
		1402	1549	1640	1909	2798
A	$\lambda_{\text{obs}} (\text{\AA})$	3373	3729.5	3938	4584.5	6739
	EW ($\text{\AA}$)	24	68	11	54	28
	z (vacuum)	1.407	1.4082	1.402	1.4026	1.409
B	$\lambda_{\text{obs}} (\text{\AA})$	3376	3728.7	Present	4582.6	Present
	EW ($\text{\AA}$)	26	70	—	55	—
	z (vacuum)	1.409	1.4077	—	1.4016	—

The differences $z_{\text{em}} - z_{\text{abs}}$ for each QSO based on Table 4 are not affected by the zero point error. They correspond to relative velocities of $2,170 \text{ km s}^{-1}$ and $2,400 \text{ km s}^{-1}$ for A and B respectively. The relative velocities each have an error of $\sim 120 \text{ km s}^{-1}$ due to the uncertainty in the emission line centres. Thus the difference in relative velocities of 230 km s^{-1} seems somewhat larger than the measuring error.

Therefore, either the absorption redshifts, or the emission redshifts may be equal, but possibly not both.

Finally, a plate was obtained on 2 April with the University of Arizona 1.5 m telescope. The seeing was relatively poor ($\sim 2.5 \text{ arc s}$), but the two images were well resolved and their measured separation was 5.7 arc s .

Discussion

The great similarity in the spectral characteristics of these two QSOs which have the same redshift and which are separated by only 6 arc s seems to constitute overwhelming evidence that the two are physically associated, regardless of the nature of their redshifts, and we do not think that a useful *a posteriori* statistical test of this assertion can be carried out. In the rest of the discussion, however, we shall assume the QSO redshifts are cosmological. The same similarities further suggest that we may be dealing with a single source which has been split into two images by a gravitational lens. We shall consider this possibility after examining the more conventional explanation involving two distinct QSOs.

In the conventional interpretation of two adjacent QSOs we must either regard it as a coincidence that the emission spectra are so nearly the same, or assume that the initial conditions, age and environment influencing the development of the QSOs have been so similar that they have evolved nearly identically. For $q_0 = 0$ and $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ the projected linear separation corresponding to $\theta = 5.7 \text{ arc s}$ is 68.5 kpc . The difference

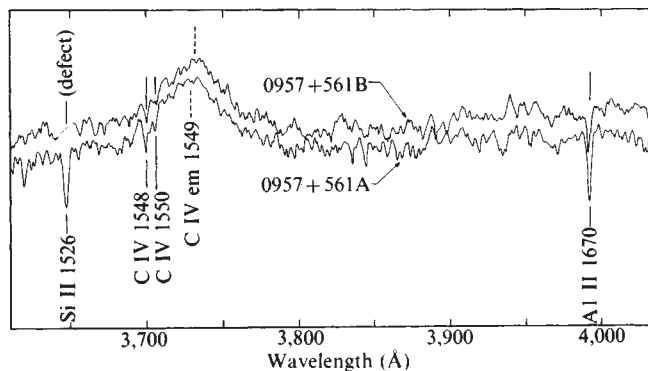


Fig. 3 Microdensitometer tracings of portions of the spectra of 0957+561 A and B. Original dispersion of the plates was $47 \text{ \AA mm}^{-1}$. The solid lines mark the position of absorption features in the two QSOs and the dashed lines mark the adopted centres of the C IV emission line.

between emission line velocities is well within the dispersion in velocities found by Stockton³ between QSOs and associated galaxies, and the masses implied by orbital motion are of the order of $10^{11} M_{\odot}$ (because of the errors in ΔV , this is more like an upper limit).

The conventional interpretation of the sources as two QSOs requires additional coincidences to explain the absorption line systems regardless of the mechanism invoked to explain the absorption. Weymann *et al.*⁴ have described three classes into which absorption systems found in QSOs in this redshift range may be placed. The first class involves ejection of material from the QSO. If the ejection of the two systems were caused by the two QSOs separately it would be an additional coincidence that the ejection velocities were so similar. If the lines arose from radial ejection by one of the QSOs, then the nearly identical redshift of the two absorption systems (the difference between which we take to be $\leq 150 \text{ km s}^{-1}$) requires a rather small angle between the direction of motion of the ejected cloud and the line of sight to the second QSO against which the cloud is projected. This implies a distance of the ejected material from the ejecting QSO of $\sim 185 \text{ kpc}$. This in turn implies exceedingly large masses and energies for the ejected material for reasonable covering factors. This argument is very similar to that made by Wolfe *et al.*⁵ for the 21 cm absorption in 3C286.

The second class of absorption involves intervening clouds associated with a cluster in which the QSOs are embedded. The velocity differences between the emission and absorption systems of A, B are typical of this class, but we must either ascribe the agreement in redshift of the two absorption systems to chance or assume that the two absorption systems are part of a common halo associated with a galaxy in the same cluster. An unusual feature if this last alternative is true is that the ionisation state is very low for this class. In the survey of Weymann *et al.* only one of about 20 absorption systems was a low ionisation system similar to those in A, B. The third class of absorption involves cosmologically distant intervening material. Neither the agreement in redshift of the systems in A and B nor their low

Table 4 Wavelengths, identifications, and derived redshifts from image-tube spectra, 2.3 m observations

Object	0957 + 561A		0957 + 561B	
	λ_{air}	z (vacuum)	λ_{air}	z (vacuum)
—	3536.4	—	—	—
Si II 1526	3648.2	1.3903	(defect)	—
C IV 1548	3699.9	1.3905	3700.1	1.3906
C IV 1550	3705.9	1.3904	3707.4	1.3914
C IV 1549 (em)	3728.9	1.4078	3732.2	1.4100
—	3835.1	—	—	—
Fe II 1608	3844.0	1.3905	—	—
Al II 1670	3992.9	1.3905	3993.6	1.3909

ionisation is then especially remarkable, but we must then ascribe to chance the fact that the intervening material happens to be at a redshift so near to the emission redshift.

We now consider the possibility that a gravitational lens is operating. The theory of gravitational imaging in a cosmological context has been considered elsewhere (see ref. 6 and refs therein) and we simply quote the main results of applying this theory. The following are the relevant parameters involved in considering the gravitational lens hypothesis: the angular separation of the images, the shape of the images and their sizes, and the amplification of the two images. There is no evidence on the plate taken on 2 April or on the POSS for any departure of the images from stellar images. The magnitude difference between A and B (Table 1) is ~ 0.3 mag and this is confirmed by our observations.

The 0.3 mag difference between the two components requires that the amplification of QSO light is ~ 4 for the brighter image, and thus implies a normal luminosity for the QSO. (This is also suggested by the absence of a strong narrow component in the CIV emission which might be expected if the source were a strongly amplified Seyfert nucleus.) The maximum angular size of the lens is only ~ 8 times that of the object, so we should not expect to resolve it on the sky.

If the matter responsible for the gravitational imaging is far from the QSO, then, from simple euclidean space calculations we estimate that at redshift z_L its mass must be $\sim 10^{13} z_L M_\odot$, and require that it be contained in a radius ≤ 30 kpc. If a galaxy is the cause, then a lower limit of $z_L \sim 0.1$ is likely from its absence on our plate material. However, the centre of such a galaxy must be within ~ 0.5 arc s of the direct line between the QSO and the observer. The chance of finding such an alignment with a massive elliptical galaxy obtained by folding in our mass requirement with Schechter's⁷ luminosity function (with a mass-to-light ratio of 30) is roughly 10^{-5} , although the precise number depends quite strongly on the magnitude differences and angular separations allowed. Thus, while such coincidences must be very rare, it is not out of the question that we should have one example in the $\sim 1,000$ QSOs known.

An apparent objection arises from the difference in the shapes of the continua between the two QSOs. It is possible that differential reddening along the two light paths may be respon-

sible. Note that the observed break at $5,300 \text{ \AA}$ corresponds to an emitted wavelength of $2,200 \text{ \AA}$ in the rest system of the QSOs. This is the wavelength of a well known resonance in interstellar extinction by dust in our Galaxy, and a model can be constructed to explain the observed continuum ratio incorporating the $2,200 \text{ \AA}$ feature at the redshift of the QSOs. This would imply that the intrinsic flux from B exceeds that from A.

Further observations would shed light on the gravitational lens hypothesis. If the flux from the object is variable, the light curves of the two images should be similar but with a relative time delay due to the difference in path lengths. The lag depends on the details of the geometry, but with the parameters discussed above would be expected to be of the order of months to years. Determination of the radio structure would also clearly be of great value.

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Since submission of this article we have heard that on 19, 20, and 21 April the two QSOs were observed by N. Carleton, F. Chaffee and M. Davis (of the Smithsonian Astrophysical Observatory) and R.J.W. using the SAO photon-counting reticon spectrograph attached to the SAO-UA multiple mirror telescope. The observations covered the range $5,900\text{--}7,100 \text{ \AA}$ with a resolution of $4 \text{ \AA}$ FWHM. Details will be reported elsewhere, but the main results are: (1) to within the measuring errors the Mg II emission lines have the same profiles and observed equivalent widths (85 and $76 \pm 12 \text{ \AA}$ for A and B respectively) and the same redshift (1.4136 ± 0.0015 for both). (2) Absorption lines due to Fe II $\lambda\lambda 2586, 2599$, Mg II $\lambda\lambda 2795, 2802$ and Mg I $\lambda 2852$ are present in both objects but are somewhat stronger in A. The mean heliocentric redshifts of the two absorption systems are 1.3915 for A and 1.3914 for B. A cross-correlation analysis confirms that the difference in the two absorption redshifts is remarkably small and corresponds to a velocity difference of $7 \pm 10 \text{ km s}^{-1}$. These observations strengthen the case for a gravitational lens.

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The moving emission features in SS433 require a dynamical interpretation

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New spectrophotometry of SS433 shows that the variable-wavelength emission features discovered by Margon et al. are due to the simultaneous presence of material having a substantial redshift and a substantial blueshift. A magnetic interpretation for the features is also ruled out by polarimetric measurements. Implications for dynamical models are discussed.

STEPHENSON-SANDULEAK 433 is an emission line object which has recently been identified as the optical counterpart of the X-ray source A1909+04 (ref. 1) and has been associated with a compact radio source and the supernova remnant W50 (refs 2-4). However, the discovery by Margon *et al.*⁵ of the highly variable-wavelength emission lines has sparked intense interest in this object. Margon *et al.* found several broad, unidentified emission features which showed daily changes in intensity, profile and wavelength, with positional changes of up

to 600 Å in 30 d. Their comprehensive analysis showed SS433 to be quite distant (≥ 3.5 kpc), luminous ($M_v \leq -3.5$), and completely stellar in appearance. Furthermore, there were strong H, He I, He II and C III–N III emission lines near rest wavelengths, indicating that the object must be relatively nearby if extragalactic. Margon *et al.* reached no conclusion as to the basic cause of the wavelength shifts for the unidentified emission lines. Accordingly, we have recently obtained spectropolarimetric and further spectrophotometric data on SS433 to determine whether the features might be due to velocity or magnetic Zeeman shifts.

Circular spectropolarimetry

We considered the possibility that a magnetic field $>10^8$ G might be capable of providing highly variable H α Zeeman emission components at the wavelengths of the observed 'red' and 'infrared' features described by Margon *et al.* This idea was supported by the recent identifications of a strong π component near $\lambda 5850$ and weaker σ components near $\lambda 7000$ in the photospheric spectrum of the magnetic white dwarf Grw+

Table 1 Identifications and velocity shifts of 'moving' features in SS433 scans

Date/instrument	Feature (Å)	Suggested identification	Redshift
2 December 1978 2.3-m	5300: (Å)	He I 5876	−0.098:
	5816	C IV rest?	—
	5945	H 6563	−0.0942
	6054	He I 6678	−0.0934
	7600:	H 6563	+0.158
3 December 1978 2.3-m (1.3-m)	5300: (5283:)	He I 5876	−0.098: (−0.10)
	5684 (5671:)	H 4681	+0.1693 (0.166:)
	5808 (5817:)	C IV rest?	—
	5914 (5922)	H 6563	−0.0988 (−0.0976)
	6834: (6848:)	He I 5876	+0.163: (+0.165:)
	7630: (7600+)	H 6563	+0.1626(−)
27 February 1979 2.3-m	4505:	H 4340	+0.0380
	5045	H 4861	+0.0378
	5810	C IV rest?	—
	6100	He I 5876	+0.0381
	6375:	Unidentified	—
	6634	Unidentified	—
	6809	H 6563	+0.0375
	6959:	He I 6678?	+0.0420:
	4515:	H 4340	+0.0403:
	5052	H 4861	+0.0392
28 February 1979 2.3-m	5419:	He II 5411 rest?	—
	5814:	C IV rest?	—
	6109	He I 5876	+0.0396
	6386:	Unidentified	—
	6628	Unidentified	—
	6812	H 6563	+0.0379
	6955	He I 6678?	+0.0415:

Colons or double colons after an entry indicate values that are uncertain or very uncertain.

70°8247 (J.R.P.A., H. S. Stockman, E.K.H. and J.L., unpublished). According to the theory, the 'infrared' feature in SS433, which has previously been seen⁵ in the range 7,000–7,600 Å, would be expected to show a strong circular polarisation signature even if the continuum polarisation proved to be much weaker. This hypothesis can be tested by circular spectropolarimetry.

These observations of SS433 were obtained on 2 and 3 December 1978 UT using the Steward 2.3-m reflector, Cassegrain spectrograph and intensified Reticon detector system in conjunction with circular polarisation analysers. The analysers consisted of quarter wave plates constructed from multiple layer interference filters followed by sheet polaroid orientated to transmit opposite senses of circular polarisation over the relevant wavelengths. The analysers were placed ahead of the 5 arc entrance aperture. The object was observed alternately through the first aperture, then the second, in the manner of a single channel polarimeter. Differing wavelength transmission properties of the polaroids were removed by observation of a continuum lamp.

The scans were obtained with the object about three hours west of meridian for 40 and 20 min total integration times, respectively, on the two evenings. Figure 1 shows in *a* and *b*, the total intensity spectra for each night; in *c*, a division of the two total intensity spectra; and in *d* a division of the spectra for 2 December having opposite circular polarisations. Some variable emission features similar to those previously discussed⁵ are marked in the 2–3 December spectra (*a*, *b*). However, we find no evidence for these or any other features in the circular polarisation spectrum. Preliminary broad-band circular polarisation measurements (on 27 November 1978) suggested that the overall optical polarisation did not exceed 0.1%. The lines and continuum could conceivably be depolarised by a mechanism such as electron scattering or propagation through a strongly magnetised plasma. However, as Fabian and Rees⁶ have pointed out, there is also a severe size/brightness temperature constraint for the required emission luminosity.

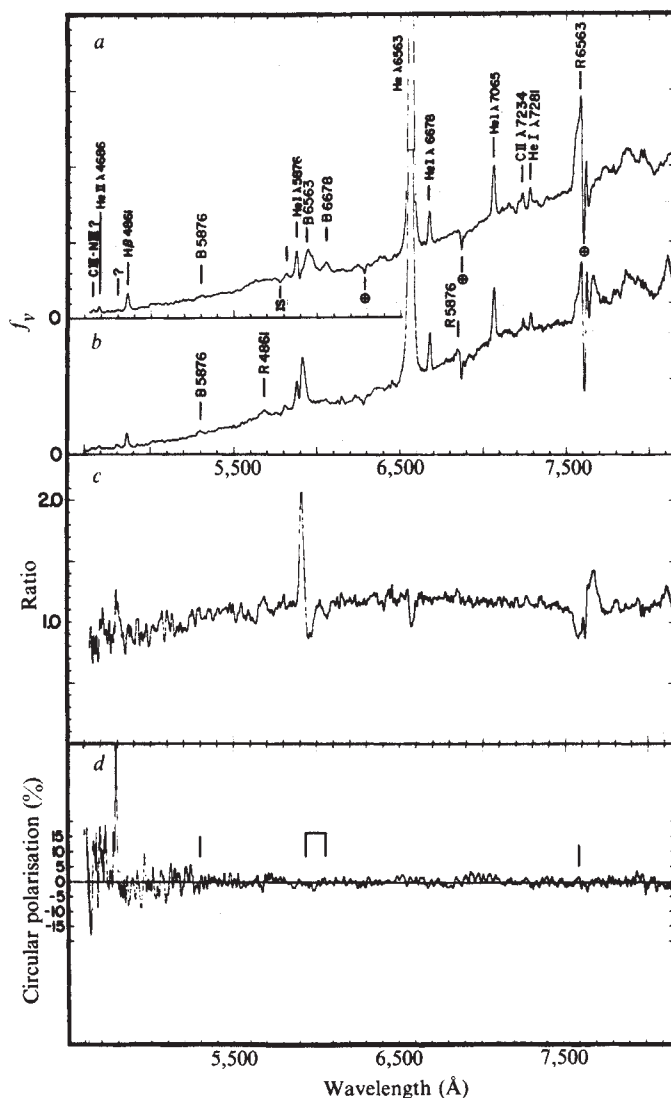


Fig. 1 Steward intensified Reticon spectrophotometry of SS433 on 2 and 3 December 1978. *a*, *b*, Total intensity spectra for each night. The rest system emission lines are identified as are emission features identified with either the redshift (R) or blueshift (B) systems; the labels correspond to the rest system wavelengths. Strong terrestrial and interstellar absorption is indicated. Thus R6563 is identified with the Margon *et al.*⁵ "infrared", and B6563 is their "red" feature (see Table 1). *a*, 2 December; *b*, 3 December. *c*, Wavelength changes in the moving features between the two nights are shown by dividing the 3 December Steward scan by the 2 December scan. *d*, Circular spectropolarimetry for 2 December, as described in the text. Positions of the principal moving features are indicated.

This constraint rules out the magnetospheres of degenerate stars observed to have high fields. A massive main sequence star magnetised to the limit in which the magnetic energy approaches the gravitational energy would be needed. In any case, the following results show that the moving emission features are not Zeeman components.

Velocity fits to the moving features

In addition to the Steward spectra of 2 and 3 December, spectrophotometric evidence was obtained using the McGraw-Hill Observatory 1.3-m and 2,000-channel intensified Reticon spectrometer on 27 November and 3 December. Further Steward spectrophotometric evidence was obtained on 27 and 28 February 1979. All but the 27 November scan show strong features similar to those described by Margon *et al.* Again the features seem to vary greatly in position and profile.

Since $H\alpha$ is expected to be the strongest line emitted by a normal composition gas over a wide range of excitations—and is very strong in the SS433 'rest' emission line system—we examined the possibility that the strong moving features similar to the 'red' and 'infrared' features of Margon *et al.* were $H\alpha$ lines from a velocity-shifted gas. The hypothesised velocities are enormous for an object suspected to be within the galaxy: some $5 \times 10^4 \text{ km s}^{-1}$ for the redshift and $2.7 \times 10^4 \text{ km s}^{-1}$ for the blueshift. Our December spectra covered much of the visible spectrum at good signal-to-noise ratio and showed weaker, variable emission features. If the velocity interpretation were correct, then their positions could be expected to match wavelengths for other lines of H, He I, He II, and so on, in the redshift or blueshift systems hypothesised for $H\alpha$. Remarkably, it was possible to account for most of the significant weak features with velocity-shifted lines of $H\beta$, He I $\lambda 5876$ and He I $\lambda 6678$ —all strong lines in the rest system. These results are listed in Tables 1 and 2.

Table 2 Mean velocity shifts from SS433 spectrophotometry

Date	Blueshift	Redshift
2 December 1978	-0.0946 ± 0.002	$+0.158 \pm 0.004$
3 December 1978	-0.0985 ± 0.001	$+0.1672 \pm 0.004$
27 February 1979	—	$+0.0383 \pm 0.0015$
28 February 1979	—	$+0.0394 \pm 0.0012$

The probability that such a good match could be made by chance is quite low. However, a definitive test of the identifications would be to show that these weaker features move as the $H\alpha$ components move. Unfortunately, the weakness of the features and the blending with atmospheric bands prevented us from making a conclusive test for the 2–3 December data.

When the object was re-observed on 27 February 1979 (Fig. 2), a very strong infrared feature was found quite close to $H\alpha$ ($\sim 6,800 \text{ \AA}$), affording an uncluttered test of our hypothesis of a redshift system. Indeed, strong features with corresponding small velocities appeared redwards of $H\beta$, He I $\lambda 5876$ and probably $H\gamma$ (Table 2). Moreover, the features have similar, complicated profiles, suggesting extended double peaks with skewed wings to longer wavelengths. This similarity is, of course, expected if they come from the same gas. On 28 February, no statistically significant shifts in these features had occurred. However, their profiles all appeared distinctly sharper; identification of corresponding He I $\lambda 6678$ and $\lambda 7065$ lines in these spectra is confused by blending.

Surprisingly, there was little evidence for a well separated blueshift system on 27 and 28 February. There are much weaker, unidentified features present which might be blueshifted $H\alpha$; but searches for corresponding $H\beta$ /He I features are frustrated by their weakness. Extended wings and structure on the short-wavelength sides of the rest $H\alpha$, and $H\beta$ suggest that a strong blue system might just be emerging near zero velocity. It is of interest to compare these scans with that published by Clark and Murdin¹; the latter showed an unidentified feature at $\lambda 6490$ which may be blueshifted $H\alpha$ but no

evidence for a redshift system, at least out to $\lambda 6800$ where the scan ends.

Implications

We have shown that the features in our data are due to hydrogen and neutral helium lines undergoing very large velocity (and perhaps gravitational) shifts. Margon, Ford and Katz (unpublished) have independently reached a similar conclusion concerning their spectrophotometry. A detailed discussion of alternative models^{6–9} should be deferred until a comprehensive set of observations is available. However, the following remarks should be relevant.

(1) The velocity-shifted gas clearly is not very hot: it is near the ionisation temperature of He I and H, but not He II. Apart from the lack of He II $\lambda 4686$ in the moving system, there is no

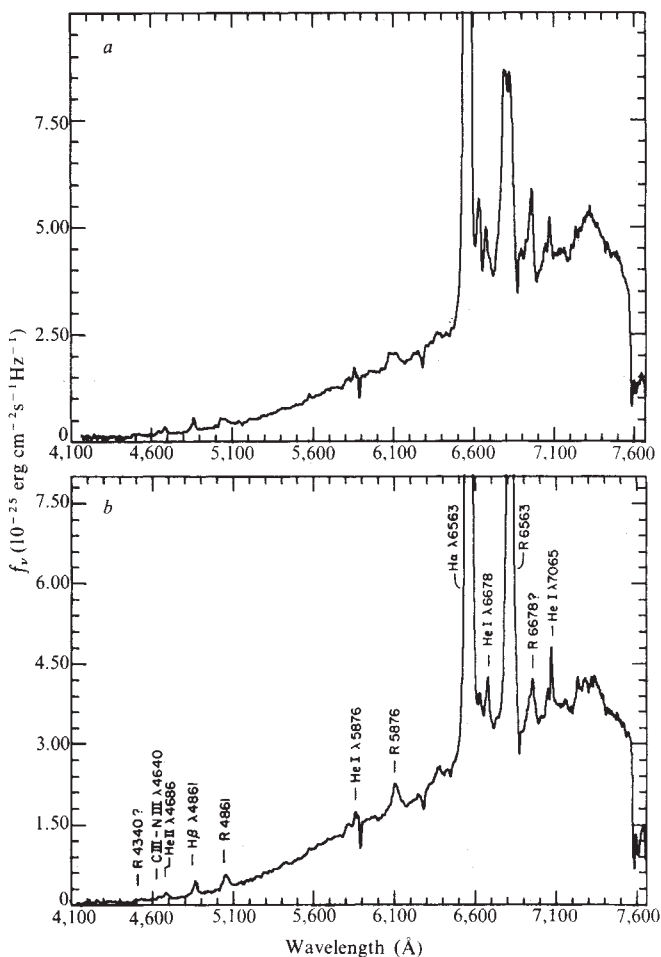


Fig. 2 Steward spectrophotometry for 27 February (a) and 28 February (b). The peak of the $\lambda 6812$ redshifted $H\alpha$ feature for 28 February does not appear on the truncated plot but lies at ~ 3.8 times the nearby continuum intensity and at ~ 0.37 of the $H\alpha$ rest line peak. Labels on the 28 February plot follow the Fig. 1 format.

evidence for forbidden lines such as [O I] $\lambda 6300$, [O III] $\lambda 5007$, and [O II] $\lambda 7319$, 7330.

(2) While the moving features may sometimes appear quite narrow (for example the blueshifted $H\alpha$ line on 3 December is $50 \text{ \AA}$ FWHM), they may also appear with complicated profiles. Note that the redshifted features on 27 February have sharp violet edges but are skewed to longer wavelengths away from the rest wavelengths; quite the opposite would normally be expected for a very thin, limb-brightened disk⁷.

(3) If the blueshift system is assumed to be absent or near zero velocity on 27–28 February, while a strong redshift system is present at $z \sim 0.04$, several implications are evident: a very small inclination angle would be required for a black hole

accretion disk model if the gravitational redshift term is to equal the velocity blueshift for gas at only a few Schwarzschild radii. Previously, Terlevich and Pringle⁷ fitted a moderate inclination angle (45°) to the Margon *et al.* features for 23–26 October 1978. For a double ejection model, there is a different inference: since the Margon *et al.*⁵ paper and our earlier data suggest that the high velocity gas is generally seen to accelerate, the positive-velocity gas on 27–28 February would apparently have been ejected somewhat earlier than the negative-velocity gas (regardless of the light travel times between ejected systems). Alternatively, the transverse Doppler shift could produce a displacement to longer wavelengths for both velocity-shifted components if the total velocities were $\sim 0.2\text{--}0.3\ c$. Finally, we discount the possibility that the unidentified emission line at $\sim \lambda 6630$ is the blueshifted H α component at a position longwards of rest H α because it is much weaker and narrower than the $\lambda 6810$ redshifted component.

We thank Drs Bruce Margon and Holland Ford for discussions on their material on SS433, including unpublished results. We also thank H. S. Stockman, S. Starrfield, P. A. Strittmatter and R. J. Weymann for stimulating discussions. John McGraw helped us appreciate the severe difficulties of a magnetic interpretation. Mrs D. Rautenkranz was responsible for the data reduction. This work was supported by NSF grants AST 76-22273, AST 78-22714, and AST 76-17600.

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Note added 26 April 1979: Two important new developments from optical spectrophotometry have occurred since this paper was first submitted.

(1) Margon *et al.*¹⁰ have reported that the line positions for both the blueshifted and redshifted systems recur with period 160 ± 3 d, or an integer multiple thereof. The two systems seem to reach maximum positive and negative velocities of $+50,000$ and $-30,000\ \text{km s}^{-1}$; the minimum velocities have not been determined. These conclusions are consistent with recently obtained Steward velocity points (which were included in the UCLA data base).

(2) High resolution ($\sim 2.5\text{--}5\ \text{\AA}$) scans obtained on 4, 5 and 6 April 1979 and similar data obtained on scattered nights during March confirm that strong blueshifted absorption lines often occur near the H β , H γ and He I $\lambda 4471$ emission lines of the 'rest wavelength' system; the scans show that the absorption lines are highly variable in strength from night to night. He II $\lambda 4686$ absorption is not seen. These sporadic 'P Cygni' profiles suggest the ejection of cool gas with $\approx 1,000\ \text{km s}^{-1}$ velocity widths from the region producing the rest emission lines. The rapid variability indicates that this region has a size of the order of one light day. Such a scale is not readily compatible with a massive black hole model for the system.

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Biologically active phorbol esters specifically alter affinity of epidermal growth factor membrane receptors

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TPA (12-O-tetradecanoyl-phorbol-13-acetate) reversibly inhibits the binding of ^{125}I -labelled epidermal growth factor (EGF) to treated mouse and human cells, but does not affect the binding of various other ligands to their membrane receptors. It alters the affinity of the receptors for EGF without changing the total number of available receptors per cell. Those phorbol esters which stimulate cell growth in culture and have tumour-promoting activity in vivo alter the EGF-receptor affinity, while the biologically inactive derivatives fail to change the affinity of EGF for its receptors.

TUMOUR PROMOTERS are compounds which are not themselves carcinogenic but which can induce tumours in mice previously treated with a suboptimal dose of certain chemical carcinogens¹⁻⁴. One of the most potent tumour promoting agents is TPA (12-O-tetradecanoyl-phorbol-13-acetate), initially isolated from croton oil⁵. Tumour promoters modulate an array of biochemical functions in mouse skin, including the stimulation of DNA, RNA and protein synthesis⁴. TPA also evokes various biological and biochemical changes when added to cultured cells, including the stimulation of DNA synthesis^{4,6,7} and cell proliferation^{4,6-8}, induction of plasminogen activator⁹

and ornithine decarboxylase¹⁰, loss of surface-associated fibronectin¹¹, either inhibition or stimulation of differentiation¹⁻⁴, and alteration in cell permeability¹².

The amphiphatic nature of the TPA molecule, and several other lines of evidence, suggest that the initial site of action of TPA and other related promoting agents is the membrane of the target cell^{2,3,12-15}. The nature of the biological and biochemical responses initiated by TPA *in vivo* as well as *in vitro*, has many similarities with those of growth stimulating polypeptide hormones such as epidermal growth factor (EGF)¹⁶ and sarcoma growth factor (SGF)¹⁷.

The above considerations led to the investigation of the effects of TPA and related tumour promoters on the interactions between EGF and its membrane receptors. A recent report by Lee and Weinstein describes the effect of TPA on epidermal growth factor binding to HeLa cells¹⁸. Their data indicate that TPA reduces the number of EGF receptors available for binding the ligand¹⁸, whereas the studies described here suggest that TPA reduces the binding of EGF to its receptors by reducing the affinity of the EGF receptor-ligand interaction, while not affecting the total number of available EGF receptors. There is also a good correlation between the degree of modulation of the EGF-receptor interactions by the various derivatives of phorbol and their ability to act as promoters of carcinogenesis both in cell culture and in mouse skin.

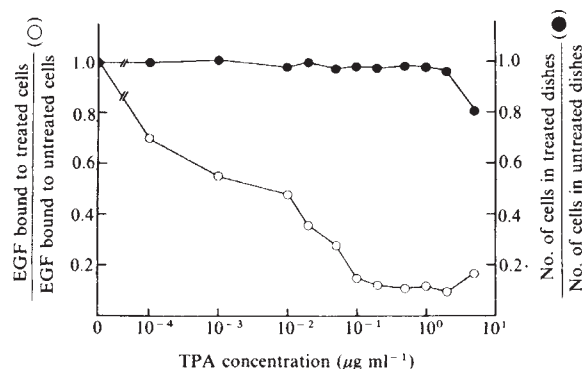


Fig. 1 Effects of TPA concentration on the binding of ^{125}I -EGF to 3T3 cells. Binding assays were performed on subconfluent cell culture monolayers in 35-mm cluster dishes. 1.3×10^5 cells were seeded per well in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (CS) 48 h before the binding experiment. The medium was aspirated, cells were exposed to growth medium containing 0–5 $\mu\text{g ml}^{-1}$ TPA dissolved in dimethylsulphoxide (DMSO). All the dishes contained 3 ml of medium, and a final concentration of 0.1% DMSO. Treated and untreated cells were incubated for 2 h at 37°C; four dishes were used for each concentration of TPA. At 2 h after TPA exposure, the cells were washed twice with 2-ml aliquots of binding buffer consisting of DMEM containing 1 mg ml^{-1} bovine serum albumin (BSA) and 50 mM *N,N*-bis-(2-hydroxyethyl)-2-aminoethane-sulphonic acid (BES) adjusted to pH 6.8. The binding assays used 2 ng of ^{125}I -labelled mouse EGF, containing $\sim 98,000$ d.p.m., in 1 ml of binding buffer. After incubation for 40 min at 37°C, unbound ^{125}I -EGF was removed and cells washed four times with 2-ml aliquots of cold binding buffer. Cells from one dish in each group were removed, using 2.5 ml of trypsin-EDTA, and an aliquot used to determine cell number in a Coulter counter. The cells were solubilised in 0.75 ml of lysing buffer (0.01 M Tris-HCl pH 7.4 containing 0.5% SDS and 1 mM EDTA) and the dishes washed twice more with 0.5 ml portions of lysing buffer. Radioactivity was determined using a Beckman gamma counter. Nonspecific binding was determined by estimating the amounts of cell-bound radioactivity in the presence of 10 μg of unlabelled EGF. All data were corrected for nonspecific binding¹⁹. About 50–100 d.p.m. above machine background bound nonspecifically to 1×10^6 cells. Untreated control dishes contained 5.1×10^5 cells and bound 5,154 d.p.m. per 10^6 cells.

TPA treatment of cells and EGF receptors

TPA elicited one round of cell division in confluent BALB/3T3 cells. This effect was observed at TPA concentrations of 0.1–0.5 $\mu\text{g ml}^{-1}$. When sparse cultures of 3T3 were treated with the optimal doses of TPA, they reached a saturation density two times higher than untreated cells although the doubling time, before confluence, of both the treated and untreated cells was the same. The untreated BALB/3T3 cells had a regular growth pattern as a flat monolayer, whereas treated cells were more refractile, exhibited a less discernible overall growth pattern, crisscrossed randomly, and showed some tendency to grow over one another. The TPA-induced changes in morphology and growth behaviour were reversible; when the treated cells were subcultured in the absence of TPA, their morphology and final cell density were not recognisably different from the untreated controls. Some of these effects on cell cultures, including BALB/3T3, have been previously described^{2,4,6,7}.

Figure 1 shows the effect of various concentrations of TPA on the binding of ^{125}I -labelled EGF to BALB/3T3 cells in culture. Cells in the log phase of growth were tested for binding 48 h after subconfluent seeding. Cells were treated with 0–5 $\mu\text{g ml}^{-1}$ of TPA for 2 h before the binding of ^{125}I -EGF. Incubations were for 40 min at 37°C, at an EGF concentration of 2 ng ml^{-1} . Exposure of cells to TPA markedly reduced the binding of ^{125}I -EGF. A maximal reduction, approximately 90%, was observed at TPA concentrations of 0.2–2 $\mu\text{g ml}^{-1}$ (0.32 – 3.2×10^{-6} M), while 50% inhibition occurred at approximately 2–

4 ng ml^{-1} of TPA (3.2 – 6.4×10^{-9} M). TPA exhibited slight toxicity at the highest dose used in this experiment. We also exposed BALB/3T3 cells to various concentrations of TPA for 70 h and 138 h before measuring EGF binding; the shape of the dose-response curves in both cases (data not shown) remained essentially the same as those obtained when cells are exposed to TPA for only 2 h. The maximum effect was observed 1–2 h after exposure, but substantial inhibition (>50%) is seen within minutes.

Figure 2 shows the comparative binding of labelled EGF to TPA-treated cells (0.1 $\mu\text{g ml}^{-1}$, 2 h) following the removal of TPA by washing as a function of time after the addition of TPA-free media, EGF bound progressively more to cells with the increasing times after the removal of TPA. Two days after replacement of TPA-containing media with TPA-free media, previously treated cells bound almost the same amount of EGF as control cells not exposed to TPA. It is impossible to completely remove TPA from the cell surface by washing with media because TPA is a highly lipid-soluble compound and rapidly interacts with plasma membranes^{2,4}. The slow reversal of the TPA-induced modulation of EGF binding may be related to the lipid soluble nature of TPA.

Generality of TPA modulation of EGF binding

We investigated the effects of TPA exposure on the binding of ^{125}I -labelled EGF (2 ng ml^{-1}) to various normal cell lines derived from mouse, rat, mink, cat, hamster, rabbit, human and chicken and to two murine cell lines transformed by the DNA tumour viruses, simian virus 40 (SV40) and polyoma. Mouse

Table 1 Specific reduction of EGF binding produced by TPA

^{125}I -ligand	Cell	^{125}I binding (d.p.m. per 10^6 cells)	
		Control	TPA-treated
Expt 1 EGF	Murine 3T3	2,947 $\pm$ 8	508 $\pm$ 21
	Con A	15,682 $\pm$ 861	16,454 $\pm$ 767
	MSA	334 $\pm$ 29	369 $\pm$ 38
	gp70	6,128 $\pm$ 569	6,048 $\pm$ 610
	Insulin	795 $\pm$ 37	880 $\pm$ 71
Expt 2 MSA	Rat kidney fibroblasts (49F-MSV)	1,619 $\pm$ 11	1,933 $\pm$ 18
	NGF Human melanoma (A875)	10,416 $\pm$ 536	10,054 $\pm$ 195
	LDL*	10,543 $\pm$ 890	10,426 $\pm$ 257

Expt 1: The binding assays were performed in triplicate as in Fig. 1. The ligands were tested on monolayers containing $\sim 5 \times 10^5$ BALB/3T3 cells per 35-mm dish 40 h after seeding. Incubations were performed in binding buffer at 37°C. Cells were treated with TPA (0.1 $\mu\text{g ml}^{-1}$) for 2 h at 37°C before binding assays. The individual ligands used are as follows: mouse EGF, 2 ng of ^{125}I -labelled peptide containing $\sim 8.5 \times 10^4$ d.p.m. and 10 μg of unlabelled material to correct for nonspecific binding; Con A, 10 ng radiolabelled ligand containing $\sim 6.6 \times 10^5$ d.p.m. and 14.5 mg of α -methyl-D-mannoside²¹ to correct for nonspecific binding; MSA, 1.0 ng of ^{125}I -labelled protein containing 1.9×10^4 d.p.m. and 4 μg of unlabelled material, to correct for nonspecific binding; gp70, 10 ng of radiolabelled protein containing $\sim 2.8 \times 10^5$ d.p.m., the nonspecific binding was corrected as described previously²⁵; bovine insulin, 2 ng of ^{125}I -labelled protein containing 4.33×10^5 d.p.m. and 20 μg of unlabelled protein to correct for nonspecific binding. Expt 2: The binding assays were done as in expt 1. The individual ligands used were: MSA, 1.5×10^6 cells per 35-mm dish and 2.0 ng of ^{125}I -labelled protein containing 1.63×10^4 d.p.m. and $\sim 8 \mu\text{g}$ of unlabelled material to correct for nonspecific bindings; NGF, approximately 1×10^6 cells per 35 mm dish and 1.25 ng of ^{125}I -labelled containing 3.63×10^4 d.p.m. and about 5 μg of unlabelled material to correct for nonspecific binding; LDL, 0.70×10^6 cells per 60-mm dish and 2 μg of ^{125}I -labelled protein containing 2.91×10^5 d.p.m. and 500 μg of unlabelled material to correct for nonspecific binding.

* Binding assays were done in duplicate.

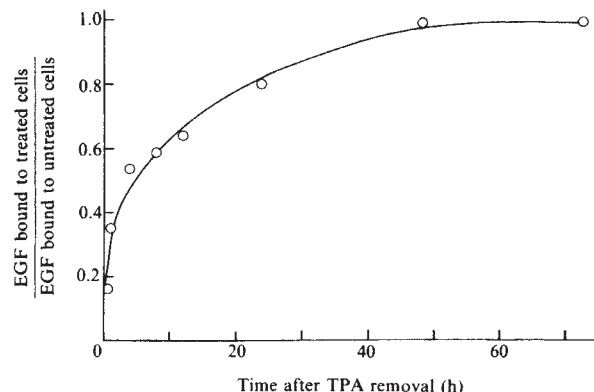


Fig. 2 Reversal of TPA effects. Approximately 1×10^5 cells per 35-mm dish were seeded. At 42 h after seeding, cells were divided into two groups. Cells in the first group were exposed to the growth medium containing $0.1 \mu\text{g ml}^{-1}$ TPA and those in the second group were exposed only to the growth medium. The media were removed 2 h later and cells washed twice with 3 ml of growth medium and fed with 5 ml of growth medium. After various intervals, binding of ^{125}I -EGF to cells of both groups was determined as described in Fig. 1. At zero time, control dishes contained 2.97×10^5 cells and bound 4,413 d.p.m. per 10^6 cells.

sarcoma virus-transformed cells were not tested as they already produce an EGF-related polypeptide and do not have available EGF receptors¹⁹. Approximately 1.2×10^5 cells of each cell type were seeded in 35-mm dishes. Specific binding of ^{125}I -EGF to the cells was determined 48 h after seeding as described in Fig. 1. Cells were exposed to $0.1 \mu\text{g ml}^{-1}$ of TPA for 2 h at 37°C before binding assays.

TPA inhibition of EGF binding was demonstrated in all types of cells investigated. Murine BALB/3T3-A31 clone 7 cells bound 3,616 d.p.m. of ^{125}I -EGF per 10^6 cells and TPA exposure of these cells reduced the EGF binding to 560 d.p.m. per 10^6 cells. SV40-transformed SV-A31-clone 6 cells derived from BALB/3T3-A31 cells bound comparable amounts of ^{125}I -EGF (4,343 d.p.m. per 10^6 cells) and TPA treatment of these transformed cells decreased the EGF binding to 489 d.p.m. per 10^6 cells. Thus, the degree of TPA induced inhibition of EGF binding was approximately the same in both the normal and

Table 2 Effects of temperature on the TPA inhibition of the binding of ^{125}I -EGF to cells

Cells	Temp of TPA-treatment ($^\circ\text{C}$)	Temp. of EGF binding assay ($^\circ\text{C}$)	^{125}I -binding (d.p.m. per 10^6 cells)		
			Control	TPA-treated	% Inhibition
Mouse	0	0	587	542	7.7
BALB/3T3-	37	0	447	57	87.2
A31	37	37	3,130	657	81.0
clone 7	0	37	2,487	440	82.3
Mink	0	0	1,530	1,533	0
Lung	37	0	1,501	245	83.7
Mv1Lu-64	37	37	4,425	725	83.6
	0	37	3,517	486	86.2

Binding assays were performed as described in Fig. 1. Cells were treated with $0.1 \mu\text{g ml}^{-1}$ TPA for 2 h at 37°C before binding assay. ^{125}I -EGF (2 ng) (78,000 d.p.m. and 38,310 d.p.m. for mouse and mink cells, respectively) was used for binding. There were 4.1×10^5 murine cells per dish 48 h after seeding and 2.9×10^5 mink cells per dish 21 h after seeding at the time of the binding assays.

SV40-transformed murine cells. Of the two normal human cell strains tested, the TPA inhibition of EGF binding was more pronounced in CRL 1224, a skin fibroblast (85% inhibition), than in CCL 137, a lung fibroblast strain (26% inhibition). The rat epithelial cell clone 52E, derived from normal rat kidney cells, binds about five times more EGF than the rat fibroblastic clone 49F, derived from the same stock of normal rat kidney cells²⁰; TPA exposure, however, affected both the fibroblastic and epitheloid cells. Thus, the action of TPA on EGF receptors seems to be a general property of vertebrate cells in culture and is not restricted to untransformed cells or to rodent cells.

Specificity of TPA inhibition of EGF binding

To test the specificity of the effect of TPA treatment on membrane receptor binding, we studied various ligands which are known to bind to cell membranes. Concanavalin A (Con A), multiplication stimulating activity (MSA), insulin, murine type C ecotropic viral glycoprotein (gp70), nerve growth factor (NGF) and low density lipoprotein (LDL) also specifically bind to cell membranes of normal and transformed cells²¹⁻²⁵. As shown in Table 1, the exposure of cells to TPA did not reduce the binding of Con A, MSA, insulin, gp70, NGF or LDL to cells, whereas in the same conditions the binding of EGF was reduced to 17% of the untreated cells.

Effects of temperature on TPA-induced reduction in the binding of EGF to its receptors are summarised in Table 2. The level of EGF binding to both treated and untreated murine or mink cells was similar when TPA treatment and the EGF binding assays were performed at 0°C . However, both TPA treatment at 37°C and EGF binding at 0°C , and TPA treatment at 0°C followed by EGF binding at 37°C , markedly reduced EGF binding in both cell systems. TPA-induced inhibition of EGF binding was maximal when both TPA treatment and EGF binding assays were performed at $20-22^\circ\text{C}$ (unpublished data). Thus, the modulation of EGF binding to its receptor which is elicited by TPA is a temperature-dependent phenomenon.

TPA exposure does not reduce the number of available EGF receptors

The effect of EGF concentration on the extent of EGF binding to control cells and TPA treated cells is shown in Fig. 3. These lines were tested for EGF binding either after a 2-h pretreatment period with TPA (mouse BALB/3T3 cells and mink CCL64 cells), or simultaneously with TPA treatment (HeLa human cervical carcinoma cells). The inhibitory effects of TPA were much greater at lower concentrations of ligand. As the

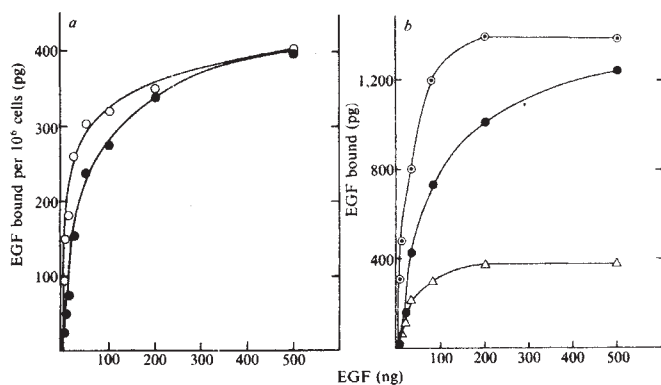


Fig. 3 *a*, Effects of EGF concentration on binding to control and TPA-treated (2 h, $0.1 \mu\text{g ml}^{-1}$) murine cells. Binding assays were performed 40 h after seeding the cells (2×10^5 per dish). Up to the concentration of 2 ng, only labelled EGF (7.8×10^4 d.p.m. per 2 ng of ^{125}I -EGF) was used; beyond this concentration unlabelled EGF was added to bring the total EGF to indicated concentration. Specific binding was measured after a 40 min incubation at 37°C ; $\circ$, control, untreated cells; $\bullet$, TPA-treated cells. *b*, Effects of EGF concentration on binding to control and TPA (2 h, $0.1 \mu\text{g ml}^{-1}$) and SGF (2 h, $100 \mu\text{g ml}^{-1}$) treated mink lung cells. The binding assays were performed as in (*a*) except that only labelled EGF was used at each concentration. $\circ$, Control untreated cells; $\bullet$, TPA-treated cells; $\triangle$, SGF-treated cells.

concentration of EGF was increased, decreasing the ratio of receptors to EGF molecules, the TPA-elicited inhibitory effects lessened or vanished at an EGF concentration of 500 ng ml^{-1} in the case of murine cells. At this concentration, the same quantity of EGF bound both to the TPA-treated cells and to the untreated cells (Fig. 3a). Figure 4 shows Scatchard plots of the effects of EGF concentration on the degree of TPA inhibition of EGF binding to HeLa cells and mink lung cells, respectively. These data produced curvilinear Scatchard plots, indicating the presence of a receptor population in both cell types with varying affinities for EGF. TPA-treated cells showed a marked decrease in ^{125}I -EGF binding at the lower concentrations of added ligand. However, the differences between TPA-treated cells and control cells decreased as the concentration of EGF increased (Fig. 4). In contrast, the treatment of mink lung cells with SGF reduced the binding of EGF to its receptors by decreasing the number of available receptors (Fig. 4b). The Scatchard plots for control and SGF-treated mink cells are almost parallel. At saturating concentrations of EGF approximately 5.5×10^5 molecules bound per mink lung cell compared with 4.1×10^4 bound per BALB/3T3 cell and 5.5×10^5 bound per HeLa cell. HeLa, a human carcinoma cell line and Mv1Lu, a mink lung cell line are of epithelial origin whereas BALB/3T3 cells are of fibroblastic origin. As in the rat kidney-cell system, epithelial cells have considerably more receptors than fibroblastic cells²⁰.

The major murine leukaemia virus glycoprotein, gp70, has high affinity receptors on BALB/3T3 cells²⁵. Treatment of these cells with TPA did not alter either the apparent number of gp70 receptors or the affinity of gp70 for its receptors. Similarly, treatment of murine or rat cells, human melanoma cells, and human fibroblastic cells with TPA did not change either the apparent number of receptors or the affinity of receptors for MSA, NGF and LDL, respectively. When the amount of gp70, MSA or NGF bound to cells was plotted as a function of the ligand concentrations, identical results were obtained for the control and TPA-treated cells. Thus, reduction in receptor affinity is not a general property of TPA-treated cells.

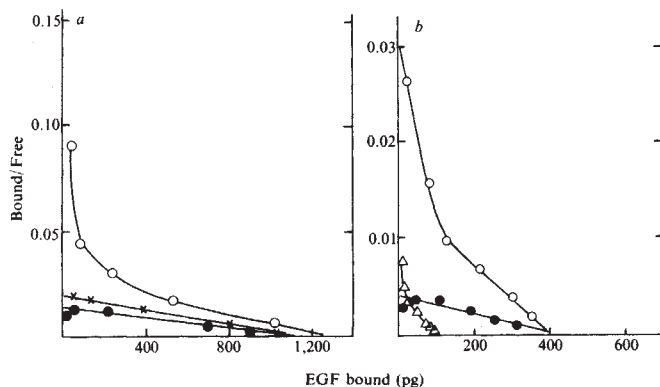


Fig. 4 a, Scatchard plots of the data on the effects of EGF concentration on the binding to control and TPA-treated HeLa cells. Binding assays were performed as in Fig. 3a except that cells were simultaneously exposed to TPA and labelled EGF. ○, Control cells; ●, 100 ng ml^{-1} TPA-treated cells; ×, 2.5 ng TPA-treated cells. b, Scatchard plots using data from Fig. 3b. ○, Control cells; ●, TPA-treated cells; △, SGF-treated cells.

Relationship between structure of tumour promoters and modulation of EGF binding

There are now available several natural and synthetic analogues of TPA with various degrees of promoting activity in the 'two-stage tumorigenesis' model¹⁻⁴. We studied their effects on EGF binding (Fig. 5). TPA was the most potent inhibitor of EGF binding among the phorbol derivatives tested. The dose required for 50% inhibition of EGF binding to cells for TPA, phorbol-12,13-di-butyrate (PDBu), phorbol-12-13-

decanoate (PDD), and phorbol-12,13-di-benzoate (PDB) were $2.9 \times 10^{-9} \text{ M}$, $6.5 \times 10^{-8} \text{ M}$, $8.6 \times 10^{-8} \text{ M}$, and $1.8 \times 10^{-7} \text{ M}$ respectively. PDA, 4α -PDD, 4-O-methyl TPA (Me-TPA) and phorbol had negligible effects on EGF binding (Fig. 5). The relative potency of these agents in the inhibition of EGF binding was: TPA > PDBu > PDD > PDB > 4α -PDD > PDA > Me-TPA > phorbol. The inhibition of EGF binding to its receptors by different phorbol derivatives correlated very well with their reported tumour-promoting activity^{1-4,26-28}.

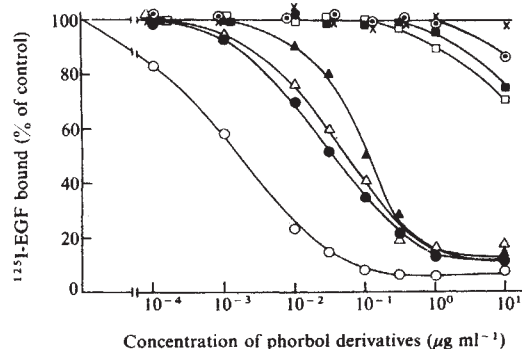


Fig. 5 Dose-response curves for various derivatives of phorbol on the binding of ^{125}I -EGF to 3T3 cells. Cells were treated with various phorbol derivatives at indicated concentrations for 2 h at 37°C before binding assays. There were approximately 5.2×10^5 cells per dish at the time of experiments 48 h after seeding. 2 ng of ^{125}I -EGF ($1.8 \times 10^5 \text{ d.p.m.}$) were used for binding. Control cells bound $7,213 \text{ d.p.m. per } 10^6 \text{ cells}$. ○, TPA; ●, PDBu; △, PDD; ▲, PDB; □, 4α -PDD; ■, PDA; ⊙, Me-TPA; and ×, phorbol.

Conclusion and implications

The EGF-competing activity of the phorbol esters parallels the tumour-promoting activity *in vivo*. The phorbol derivatives lacking tumour-promoting activity also lack EGF-competing activity. TPA treatment seems to modulate EGF binding by decreasing the affinity of the receptors on the treated cells for EGF, rather than by decreasing the number of receptors per cell. This affinity modulation is reversible and dependent on time, temperature and TPA concentration. The effect appears to be specific, for the EGF receptor system, as four other receptor-ligand systems tested in the same TPA-treated cells and three receptor-ligand systems in the other cells did not show any alterations in receptor affinity. TPA modulation of EGF binding is observed with doses of promoter comparable to those required to elicit biological responses *in vivo* as well as *in vitro*²⁹. The above data suggest that TPA-mediated alterations in growth factor(s)-receptor interactions might be related to the underlying mechanism by which tumour-promoting agents initiate a chain of events causing alterations in cellular growth and function. Interestingly, EGF has been reported to enhance the tumorigenesis induced by a chemical carcinogen³⁰. SGF, produced by mouse sarcoma virus-transformed cells also interacts with EGF receptors, stimulates cell growth and anchorage-independent growth¹⁷. The putative endogenous growth factor(s) produced in response to the exposure of cells to tumour-promoting agents, may, then, activate a programme of gene expression in those cells that have already been genetically altered by initiating agents.

There is need for rapid cell culture assays for tumour-promoting agents both of exogenous and endogenous origin. The data presented here, showing the consequences of promoter treatment on EGF-receptor interactions and the specificity, sensitivity and rapidity of this response, might provide a means for the qualitative and quantitative detection of other classes of tumour-promoting agents.

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Calcium transients and intramembrane charge movement in skeletal muscle fibres

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Myoplasmic calcium transients in response to pulse depolarisations have been monitored using the dye antipyrilazo III, which provides a linear measure of the change in cytoplasmic free calcium concentration. Intramembrane charge movements were recorded simultaneously with the calcium transients. Except for its initial phase, the calcium transient during each pulse closely followed the time course expected for calcium redistribution between three intracellular compartments with time-independent flux coefficients. The time at which the flux coefficients attained their steady values occurred just after the intramembrane charge movement had been completed. This is the required sequence if charge movement were to control one or more coefficients for calcium fluxes across the sarcoplasmic reticulum membrane.

DEPOLARISATION of a skeletal muscle fibre gives rise to a redistribution of charge within the fibre membrane¹⁻³. It has been suggested that this voltage-dependent intramembrane charge movement may occur in the transverse (T) tubule and may activate calcium release from the adjacent sarcoplasmic reticulum (SR) into the myoplasm. As the released calcium binds to regulatory sites on the contractile filaments, the inhibition of filament interaction would be removed and contractile activation would be achieved. On repolarisation the intramembrane charge returns to its initial distribution, which would turn off calcium release. Intramembrane charge movement would thus act as the voltage-sensitive step in the depolarisation-contraction coupling process^{1,2,4}.

Two observations consistent with the hypothesis that charge movement controls calcium release are: (1) that different amplitude pulses having durations which produce just detectable contractions move approximately the same amount of intramembrane charge⁵; and (2) that the repriming of the ability to produce threshold contractions during repolarisation of depolarised fibres seems to be consistent with the reappearance of the charge movement process⁶. To further investigate a possible link between intramembrane charge movement and SR calcium release, we have simultaneously monitored both the

current I_Q produced by charge movement and the free myoplasmic calcium concentration $[Ca]$ in voltage-clamped skeletal muscle fibres. The results indicate that I_Q is complete at about the time that the Ca release process appears to be fully activated. After the charge has moved, the time course of change in $[Ca]$ almost exactly follows the prediction for Ca redistribution between three compartments with time-independent transfer coefficients.

An optical signal proportional to the myoplasmic calcium concentration

Bundles of intact fibres from the semitendinosus muscle of room temperature-adapted summer frogs (*Rana pipiens*) were exposed to a high-potassium, calcium-free relaxing solution⁷. A short segment cut at both ends was obtained from an individual muscle fibre and was mounted in a double Vaseline gap chamber, each gap having a design similar to that of the single gap used previously⁷. The central pool was about 700 μ m wide and the walls separating pools were each about 250 μ m wide. Internal⁸ and external⁷ solutions were designed to minimise all ionic conductances (see figure legends). In similar conditions, cut muscle fibre segments exhibit normal contractile activation as evidenced by the strength-duration curve for just-detectable contraction⁷. All experiments were carried out at 2-4 °C.

The central pool was held at virtual ground with an operational amplifier which monitored the total current applied through one of the end pools. Voltage was recorded between the other end pool and the central pool and ranged from about 85 to 100% of the voltage applied to the current-passing pool. The membrane potential V_m was therefore taken to be about 1.05 times the gap voltage. Fibres were voltage clamped at a holding potential of -100 mV. I_Q was extracted from the total current for a depolarising pulse by subtracting both the linear capacitative current determined with hyperpolarising pulses and remnant ionic currents using a slight variation (ref. 8 and P. Horowicz and M.F.S., in preparation) of standard procedures¹⁻³. Movement was minimised by stretching all fibres to sarcomere lengths close to those giving no contractile filament overlap.

The metallochromic dye antipyrilazo III (AP III) was introduced into each fibre by diffusion from the solution bathing the two cut ends. AP III is a convenient indicator of $[Ca]$ as it reacts rapidly with calcium and changes its colour on calcium binding⁹. It exhibits a $[Ca]$ -dependent increase in absorbance at 720 nm, a wavelength at which magnesium has no direct effect on AP III

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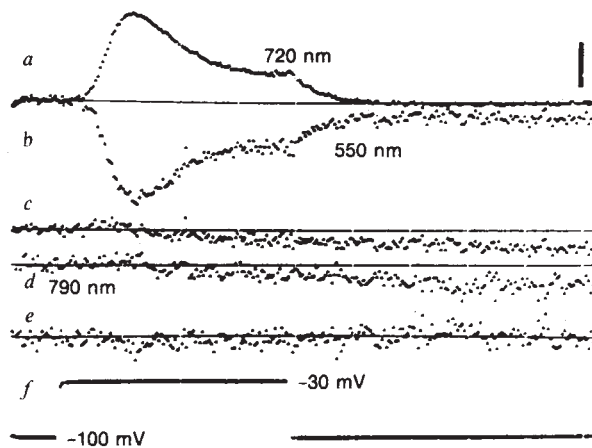


Fig. 1 Light absorbance changes at different wavelengths and linear combinations of absorbance changes produced by a given voltage clamp depolarisation of an AP-III-containing fibre. The optical records were composed of the following changes (ΔA_λ), in absorbance at wavelength λ : (a) ΔA_{720} ; (b) ΔA_{550} ; (c) $\Delta A_{720} + 0.93 \Delta A_{550}$; (d) $4.4 \Delta A_{790}$; (e) $\Delta A_{720} + 0.93 \Delta A_{550} - 4.4 \Delta A_{790}$. The calibration bar for all optical records is 4.3×10^{-3} and refers to each of the above ΔA_λ signals divided by the total fibre absorbance at $\lambda = 550$ nm. The voltage (record f) went from a holding potential of -100 to -30 mV. Pulse duration was 100 ms. Each point gives the average value of the signal during a 1-ms interval and each ΔA record is the average of two measurements. The solution in the central pool contained, in mmol l $^{-1}$: tetraethyl-ammonium sulphate, 75; Cs $_2$ SO $_4$, 5; Cs Tris-maleate buffer, 5; CaSO $_4$, 10; tetrodotoxin, 10^{-7} g ml $^{-1}$; pH 7.0. The solution in the end pools contained: caesium glutamate, 108; MgCl $_2$, 6.5; sodium Tris-maleate buffer, 4.5; caesium Tris-maleate buffer, 13.2; EGTA, 0.2; CaCl $_2$, 0.069; Na $_2$ ATP, 5; glucose, 5; AP-III, 1; pH 7.0. Both cut fibre ends were exposed to this solution for about 80 min before the measurements. AP-III from K and K Labs was used without further purification. Each wavelength was set using a 10-nm half-peak bandwidth interference filter (Ditric Optics, 3-cavity filter) in the incident light beam. All ΔA signals were obtained by amplifying and filtering the transmitted light intensity signal using a Tektronix 565 oscilloscope with a 3A9 plug-in amplifier set for 0.1 Hz to 1 kHz bandwidth. Fibre B23, 3.46 μ m per sarcomere (3°C).

absorbance, and a [Ca]-dependent decrease in absorbance at 550 nm (ref. 9). At 790 nm AP III has negligible absorbance in either the free or calcium-complexed form. Any [Ca]-dependent AP III absorbance change should thus exhibit an identical time course but different size and opposite polarity at 720 and 550 nm, whereas any absorbance change at 790 nm should be independent of [Ca] and should give the time course of light transmission changes due to fibre movement. Accordingly, changes in fibre light absorbance (ΔA) were monitored at 720, 790 and 550 nm using the optical system previously described¹⁰. In all cases the change ΔI in transmitted light intensity I was sufficiently small compared to I that ΔA , which is equal to $-0.43 \ln[(I + \Delta I)/I]$, could be closely approximated by $-0.43 \Delta I/I$. Each signal to be monitored was digitised as its average value for each of a sequence of 255 successive 1-ms intervals by means of a voltage to frequency converter and digital counter^{2,10}. A laboratory computer (Digital Equipment PDP-8E) was used to store, signal average, process and analyse all records.

Figure 1 presents ΔA traces or linear combinations thereof produced in the same fibre by a 70-mV depolarising voltage pulse (trace f). At the start of the pulse the ΔA signal at 720 nm (ΔA_{720} , trace a) began with a sigmoid foot after a latency of about 11 ms. The signal then rapidly increased to a peak after which it declined more slowly towards a steady positive level which was maintained during the pulse. On repolarisation ΔA_{720} declined monotonically. The ΔA signal at 550 nm (ΔA_{550} , trace b) followed almost the same time course as ΔA_{720} but exhibited

a decrease instead of an increase in absorbance, as expected for a [Ca]-related signal. The small signal at 790 nm (trace d) starts late and rises slowly as expected for contractile movement in these stretched preparations. Signals having qualitatively similar time courses as traces a and b have been reported for voltage-clamped intact fibres microinjected with arsenazo III^{11,12}, a calcium indicator dye which has a higher affinity for calcium than AP III in calibrating solutions⁹.

The signals at 720 and 550 nm, traces a and b of Fig. 1, were each composed of a component due to the AP III absorbance change plus a smaller component due to fibre movement. To show this, ΔA_{550} was slightly scaled down and added to ΔA_{720} . Bearing in mind that a [Ca]-dependent dye absorbance change should have an identical time course but different size and opposite polarity at 720 and 550 nm and that the movement artefact began relatively late, the factor for scaling ΔA_{550} was chosen to make the sum zero during the early part of the individual ΔA signals. This procedure should remove the dye absorbance change at all times but should not remove the movement artefact. The result, presented as trace c of Fig. 1, does indeed exhibit a small remaining deflection which begins relatively late and closely resembles ΔA_{790} (trace d). The difference (trace e) of traces c and d was zero, showing that within experimental error trace c was entirely movement artefact.

The movement artefact was relatively less important compared to the [Ca]-related signal in ΔA_{720} than in ΔA_{550} (Fig. 1, traces a and b) because the total fibre absorbance, which was predominantly due to the dye, was much less at 720 than at 550 nm. The fact that the early part of trace c could be made zero indicates that the early intrinsic decrease in absorbance observed in dye-free fibres^{10,13}, which does not change sign but becomes larger at shorter wavelengths¹⁴, must have been negligible compared to the calcium-related AP III signal in these experiments.

The movement artefact included in ΔA_{720} could always be determined by monitoring ΔA_{790} and by making use of the facts that the artefact was proportional to ΔA_{790} and that in the absence of movement, the signal at 720 nm decayed exponentially to zero (as discussed below). Once the movement artefact was determined, it could be subtracted from ΔA_{720} in the relatively few cases when it was not negligible. The remaining signal, which is the dye absorbance change, is hereafter called ΔA .

We interpret ΔA as being a linear measure of the change in [Ca]. For this interpretation to be valid the following conditions must be fulfilled: (1) ΔA should be due to calcium binding to AP III and not to other possible reactions of the dye; (2) the amount of calcium-dye complex should be a linear function of [Ca]; (3) AP III should be restricted to the myoplasm.

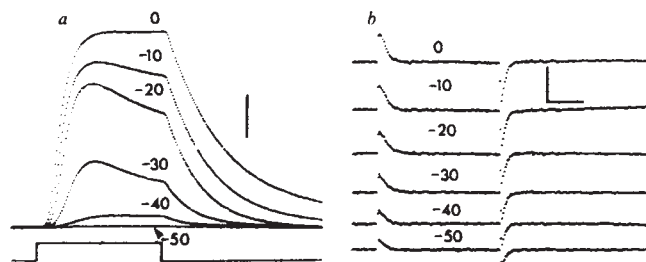


Fig. 2 Voltage dependence of calcium transients and intramembrane charge movement. a, Superimposed ΔA signals at 720 nm for 100-ms depolarising pulses to the indicated voltages V_m . Each record is the average of six determinations, with no correction made for fibre movement. The calibration bar corresponds to $\Delta A_{720}/A_{550}$ of 1.4×10^{-2} . The pulse timing is denoted by the diagrammatic construction below the records. b, Intramembrane charge movement current records for the same pulses as in (a). The vertical calibration denotes 3.2 μ A μ F $^{-1}$ and the horizontal calibration denotes 30 ms. Same fibre and conditions as in Fig. 1.

Consistent with condition (1), microinjection of calcium into AP III-containing fibres produced absorbance signals having a similar wavelength dependence as the signals produced by fibre depolarisation. Use of solutions containing relatively high EGTA concentrations in both cut end pools completely abolished the ΔA signals. Contributions due to two other possible reactions were minimised by using a relatively high pH buffer concentration in the end pools and by recording at 720 nm where Mg has no direct effect on dye absorbance. Condition (2) will be fulfilled provided that the calcium-dye reaction has one-to-one stoichiometry and that $[Ca]$ remains well below the dissociation constant for the reaction, which is 200–300 μM in solution⁹. The exponential nature of the ΔA signals (see below) for even the largest depolarisations indicates that $[Ca]$ was indeed within the range over which ΔA was acceptably close to a linear function of $[Ca]$. Finally, AP III is not removed from solution when incubated with isolated rat liver mitochondria or red cell suspensions⁹. In similar experimental conditions, no binding of AP III to isolated rabbit skeletal muscle SR vesicles could be detected (A. Scarpa, personal communication). These observations indicate that condition (3) was fulfilled. Summing up, the interpretation of ΔA as a linear measure of $[Ca]$ seems to be justified as conditions (1)–(3) appear to be fulfilled.

Calcium transients are steeply dependent on membrane voltage

Figure 2a presents superimposed ΔA_{720} records, not corrected for movement, from one fibre for 100-ms depolarising pulses to voltages increasing in 10-mV steps from -50 to 0 mV. Qualitatively similar records were obtained from 12 other fibres. The minimum detectable signal usually appeared for a depolarisation to between -50 and -40 mV. For the fibre in Fig. 2, no significant ΔA_{720} was detected at -50 mV. At -40 mV a relatively small and slowly developing ΔA signal was observed after a latency of 17 ms. As seen in Fig. 2a, both the maximum rate of rise of the ΔA_{720} signals and their peak values during a pulse increased steeply with voltage over the range from about -45 to -20 mV. Both continued to increase, but less steeply, for pulses up to +20 mV, the maximum depolarisation tested in a few fibres. The latency intervals decreased markedly between about -45 and -30 mV and then decreased less markedly with larger depolarisations. The steady levels which the ΔA_{720} signals were approaching during each pulse also increased with increasing depolarisation. The final rise of ΔA_{720} for the depolarisation to 0 mV in Fig. 2a was due to the movement artefact and was never seen in corrected (ΔA) records.

Charge movement current records for each of the pulses in Fig. 2a are presented in Fig. 2b. The charge movement in this preparation displays a similar dependence on voltage as observed with the single gap preparation^{5,8} but with slightly faster kinetics, probably due to the improved clamp speed in the present experiments. The values of maximum charge moved were consistently lower than the values from previous microelectrode¹⁻³ or single gap⁸ studies, which may be ascribed to the fact that the present fibres were stretched to more than 3.0 μm per sarcomere¹⁷. We have, however, made no systematic study of the effect of sarcomere length on charge movement in otherwise identical conditions.

It is evident from Fig. 2 that the pulse to -50 mV, which produced no detectable absorbance change, did move a significant amount of intramembrane charge. This is in agreement with the observation that a set amount of intramembrane charge must be mobilised in order to produce a detectable fibre contraction⁵.

A simple mathematical description for most of the calcium transient

Figure 3A presents ΔA signals (traces a, b and c) for 100 ms pulses (traces d) to -40, -30 and 0 mV in another fibre. Except for its initial section, each signal ΔA_{ON} during a pulse could be

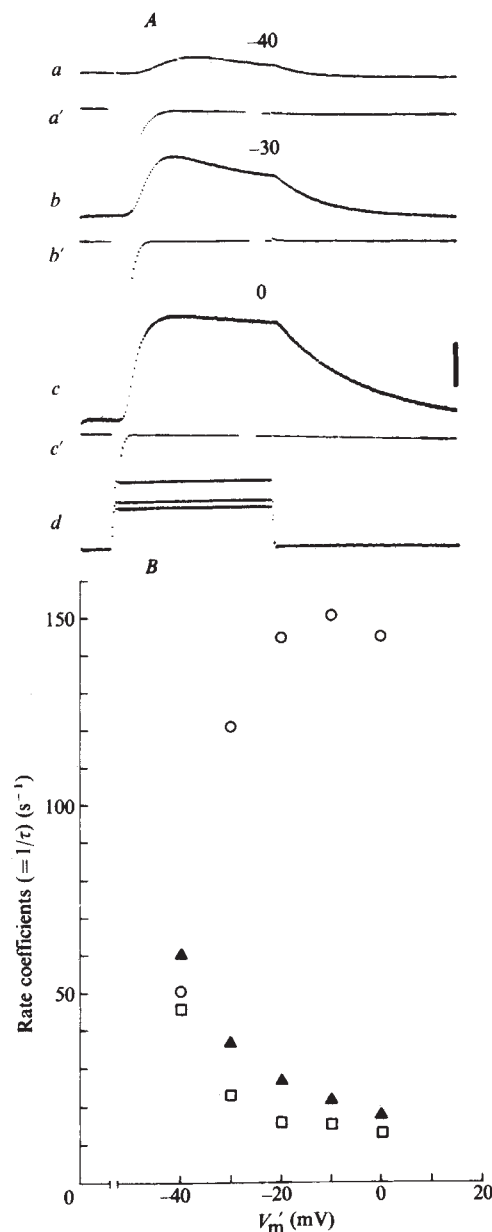


Fig. 3 Characterisation of the calcium transient's time course at various voltages. **A:** a, b and c give ΔA at 720 nm for 100-ms depolarising pulses (record d) to the voltages V_m' indicated by the number above each record. Record c was corrected for movement artefacts by adding 9.2 ΔA_{790} to ΔA_{720} . Records a, b and c are averages of two determinations. Traces a', b' and c' give the residuals remaining after subtracting each ΔA record from the best fit of equations (1) and (2) to the same record. Only points after the ON or OFF transition times s_{ON} or s_{OFF} were used in the curve fitting procedure, which was a general iterative routine for obtaining least square fits with nonlinear functions¹⁵. The residuals were then calculated for all ON and OFF points, both before and after s_{ON} and s_{OFF} . The first gap in each of the residual traces begins at the start of the pulse. It occurred because the residuals were out of range during this interval. The second gap is over before the end of the pulse. For this period the residuals, which were essentially equal to zero, were simply blanked out. This is to indicate that the ON and OFF portions of each ΔA record were fit separately using equations (1) and (2). The calibration bar corresponds to $\Delta A_{720}/A_{550}$ of 1.6×10^{-2} for both the ΔA and the residual traces. **B:** Rate coefficients giving the best fit of equations (1) and (2) to the ΔA signals in (A) after s_{ON} and s_{OFF} . $\circ$, β_1 the rate coefficient for the faster (rising) ON component; $\square$, β_2 , the coefficient for the slower (declining) ON component; $\triangle$, λ , rate coefficient for the OFF decay. The values at -20 and -10 mV were obtained from fits to ΔA records for these voltages which are not shown in (A). Same pool solutions as in Fig. 1. Fibre B24, 2.96 μm per sarcomere (2.5 °C).

closely approximated by the sum of two exponential functions of time t plus a constant so that for times following s_{ON} ,

$$\Delta A_{\text{ON}} = B_1 \exp[-\beta_1 t] + B_2 \exp[-\beta_2 t] + D_{\text{ON}} \quad (1)$$

The ON 'transition time', s_{ON} , is defined as the interval from the start of the depolarisation to the time at which ΔA followed equation (1). The fact that ΔA_{ON} followed equation (1) closely after s_{ON} is illustrated by the ON segments of traces a' , b' and c' , which give the residuals calculated by subtracting each ΔA record from the least squares fit¹⁵ of equation (1) to the same record. The time at which each of the residual traces reaches zero during a pulse determines the end of s_{ON} for that voltage. Note that s_{ON} is longer than the latency interval; it includes both the latency and the time corresponding to the initial foot of the signal which equation (1) cannot describe.

Following the pulse and at times after the OFF transition time, s_{OFF} , the absorbance signal ΔA_{OFF} declined along a single exponential time course,

$$\Delta A_{\text{OFF}} = C \exp[-\gamma t] + D_{\text{OFF}} \quad (2)$$

The fit of equation (2) to ΔA_{OFF} is illustrated by the OFF segments of the residual traces a' , b' and c' in Fig. 3A. The steady OFF level D_{OFF} was about zero after removing any movement artefact.

The goodness of all ON and OFF fits at times after s_{ON} and s_{OFF} was assessed by various criteria: (1) The variance of the residuals (σ_r^2) was comparable to the variance of the baseline before the pulse. (2) σ_r^2 For the ON and OFF agreed to within 30%. (3) σ_r^2 Was not dependent on pulse voltage. (4) The values of β_1 and β_2 or γ were quite insensitive to changes in the interval after s_{ON} or s_{OFF} used for the fit. Some sample records were also analysed after s_{ON} or s_{OFF} on a DEC-10 computer using a Fourier transform program for fitting multiple exponentials¹⁶ and were shown to satisfy the following additional criteria (nos 5-7). (5) The number of exponential components was clearly defined as 2 for the ON and 1 for the OFF. (6) The signal-to-noise ratio of fit, that is the ratio of the peak of the signal to the standard deviation of the residuals, ranged from 50 to 1,000, the smallest values corresponding to the smallest amplitude signals processed. (7) The auto-correlation of the residuals at 1- to 5-ms lag was small. These criteria clearly demonstrate that equations (1) and (2) described the records extremely well after s_{ON} and s_{OFF} .

Figure 3B shows the voltage dependence of the exponential rate coefficients determined by fitting equations (1) and (2) to the records in Fig. 3A after s_{ON} and s_{OFF} . The rate coefficient β_1 of the faster (rising) exponential term in ΔA_{ON} increased with pulse voltage whereas the rate coefficient β_2 of the slower (declining) ON exponential decreased with V'_m . β_1 and β_2 were essentially equal at about -40 mV, a slightly suprathreshold voltage. The rate coefficient γ of the single exponential term in ΔA_{OFF} was generally less than β_1 and greater than β_2 . Like β_2 , γ decreased as V'_m was increased. These findings were consistent in all fibres studied.

The calcium transients correspond to a three-compartment system

Equation (1) gives the general expression for the relaxation to steady state in each compartment of a closed three-compartment system with time-independent fractional transfer coefficients¹⁸. One possible interpretation of the very close fit of equation (1) to the ΔA time course at times after s_{ON} is that the myoplasm constituted one compartment of a three-compartment calcium exchange system and that the transfer coefficients for all significant unidirectional calcium fluxes were constant after s_{ON} . The other two compartments might be the release and uptake pools of the SR¹⁹. Each unidirectional flux would be given by the calcium concentration in the efflux compartment times a fractional transfer coefficient having the appropriate dimensions¹⁸. If some or all of the transfer coefficients were voltage dependent, a change in fibre membrane potential would produce calcium redistribution and a

ΔA signal. For example, a depolarising pulse would shift the coefficients from their resting values in such a way that calcium would move from SR to myoplasm. As there are many sets of fractional transfer coefficient values that could give rise to the same overall exponential rate coefficients, the actual values of the individual transfer coefficients could not be specified from the present data without further assumptions.

The single exponential character of the signal after s_{OFF} (equation 2) corresponds to a two compartmental relaxation and suggests the vanishing of the transfer coefficients for release with only uptake fluxes remaining operative on repolarisation. The definite dependence of the OFF exponential rate coefficient γ on the preceding pulse voltage is indicative of changes in the SR that outlast the depolarising pulse and do not appear to be reversed during the time required for ΔA to decay after the pulse. As the system closely followed equation (1) from s_{ON} to the end of the pulse, the transfer coefficient change or changes responsible for the observed voltage dependence of γ must either have been completed during s_{ON} or must not have significantly influenced the ΔA time course after s_{ON} . Evidence supporting an inhibition of SR uptake function following voltage clamp depolarisation¹¹ or tetanic stimulation²⁰ of intact fibres and during interventions that depolarise the T-tubules or the SR of skinned fibres²¹ has recently been presented. The present results support the proposal of Stephenson and Podolsky²¹ that the increase in myoplasmic calcium is due to both an increase in the release transfer coefficient and a decrease in the uptake transfer coefficient.

Steady-state SR activation may be attained just after completion of charge movement

A consequence of interpreting ΔA_{ON} in terms of a three-compartment system is that s_{ON} must equal the time required for the transfer coefficients for all significant calcium fluxes to attain their steady-state values at the new voltage. Before s_{ON} the system would not be expected to follow equation (1), because the transfer coefficients would be in the process of switching

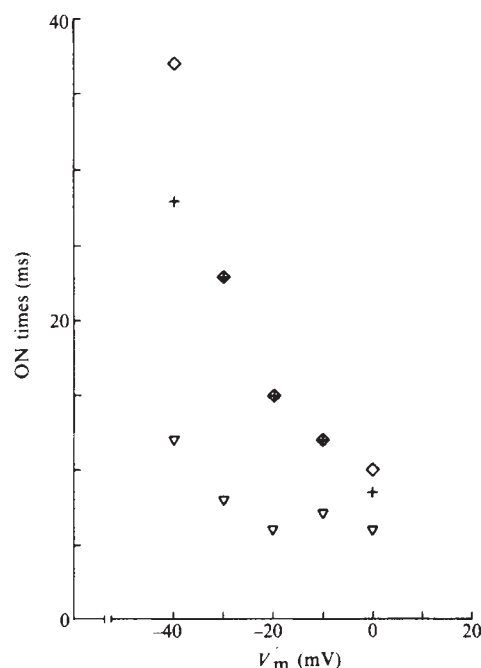


Fig. 4 ON times for the ΔA signals and for membrane charge movement. $\diamond$, ON transition time s_{ON} ; +, time required to move 98% of the membrane charge which moves at each voltage; ∇ , ON latency time, determined as the time from the start of a pulse to the first of two consecutive 1-ms intervals at which the absorbance deviated by more than two baseline standard deviations from its resting level. Same fibre and records as for Fig. 3.

from resting to new values. In the simplest situation this could be envisioned as an SR calcium 'gate opening' at the ON and a 'gate closing' at the OFF.

A possible participation of membrane charge movement in 'gating' SR calcium permeabilities was investigated by comparing s_{ON} and the time required for the intramembrane charge movement to occur. The rationale behind such a comparison is that if membrane charge movement is the voltage-dependent step controlling SR calcium release, then the transfer coefficients for calcium fluxes across the SR could not attain their steady levels until the membrane charge had reached its steady-state distribution at the new voltage. Consequently, s_{ON} should be equal to or longer than the time for membrane charge movement. Figure 4 presents times for charge movement to be 98% complete (crosses) and ON transition times (diamonds) as a function of membrane potential for the fibre of Fig. 3. In this fibre these two times agreed quite closely. For comparison, ON latency times (triangles) are also presented in Fig. 4.

A more extensive comparison of ON charge movement times and s_{ON} for several fibres is presented in Fig. 5, where the times taken for 95 and 98% of the charge to move on depolarisation are plotted against the transition time for ΔA_{ON} . These data show that equation (1) began to fit the ΔA signals at or slightly after the end of the charge movement. This is consistent with the hypothesis that charge movement controls the transfer coefficients for calcium movement across the SR membrane.

The situation was not symmetrical at the OFF. After the pulse both the charge movement kinetics were faster and the transition times were shorter than during the pulse and neither depended on the preceding pulse voltage. s_{OFF} ranged from 2 to 5 ms, a time at which the present 1-ms sampling showed that 16–86% of the charge had returned to its resting position. These results indicate that on repolarisation the fractional transfer coefficients reached their steady OFF values after only a fraction of the charge had moved back, which would be the case if several charged particles were required to gate an individual channel or if charge moved to the activating position in sequential steps. Precise characterisation of the events at pulse OFF will require better time resolution.

The insert records in Fig. 5 show the time courses of both I_Q (trace *a*) and the residuals (the fit of ΔA minus ΔA itself, trace *b*) for the same pulse to -20 mV. From trace (*a*) it is apparent that relatively small amounts of noise in an I_Q record or relatively small errors in eliminating time-dependent ionic current components from the record could give rise to relatively large errors in determining the times for 95 and 98% of the charge movement. This probably accounts for much of the scatter exhibited by the points in Fig. 5.

Alternative interpretations

Our analysis of the AP III calcium transients has been limited to the ΔA time course. Little attention was devoted to the ΔA amplitudes because the actual levels of [Ca] and calcium-dye complex corresponding to a given ΔA depend on the calcium-dye dissociation constant and on the extinction coefficients for free and calcium-complexed dye, which have not been determined for the myoplasmic environment. The time course analysis was itself restricted to times when equations (1) and (2) accurately described the ΔA signal. This restriction is reasonable if the intervals considered did in fact correspond to times when a three-compartment system was operating with time-independent fractional transfer coefficients. It must be noted, however, that alternative models and equations could probably describe more or even all of the ΔA time course. The values of s_{ON} and s_{OFF} are thus clearly dependent on our choice of model and equations. Given the close fit of equations (1) and (2) to the ΔA records, it seems unlikely that alternative equations could provide better descriptions of ΔA after s_{ON} and s_{OFF} .

Other interpretations might even be proposed to account for the observed [Ca] time course after s_{ON} . One alternative would be to identify the three compartments with the SR, myoplasm and myofilaments. This seems improbable, however, as it would

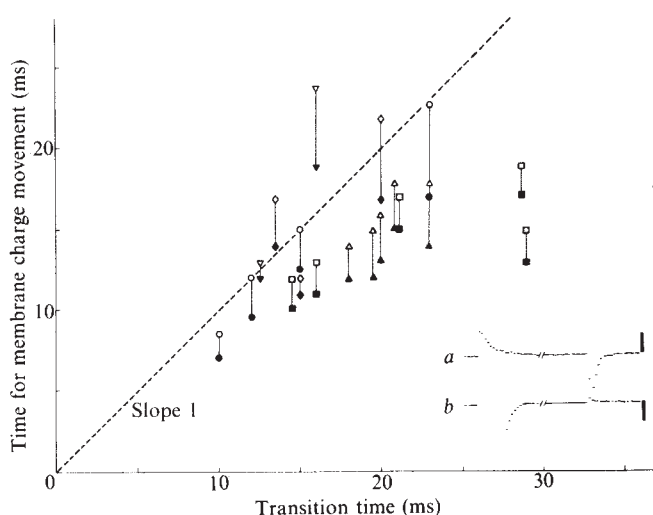


Fig. 5 Comparison of ON transition times with the time necessary for the membrane charge to move during a depolarising pulse. The times required to move 95 and 98% of the total charge which was moved during each pulse are plotted (ordinate) as the lower points (filled symbols) and upper points (open symbols) of each pair of connected points. These times were determined by integrating I_Q records such as the one in insert trace *a*. Transition times were determined from the difference between each optical record ΔA_{ON} and its best fit with equation (1) (insert trace *b* and Fig. 3A *a'*, *b'*, *c'*). The ON transition time is defined as the time between the start of the pulse and the first digitised interval where the fit was within two standard deviations of ΔA . Only results from records having 'well defined' transition times are presented in the figure. For a transition time to qualify as well defined it had to be independent of the interval chosen for fit. The same definition and criterion were also applied to determine OFF transition times but the OFF values are not plotted here. Each symbol represents results obtained from a given fibre: $\circ$, fibre B23 (see Fig. 1); $\square$, fibre B24 (see Fig. 2); ∇ , fibre B17, 3.36 μm per sarcomere, 2 °C; $\triangle$, fibre B19, 3.29 μm per sarcomere, 3 °C; $\diamond$, fibre B27, 3.49 μm per sarcomere, 3 °C. Fibres B17, B19, B23 and B24, same pool solutions as Fig. 1. For fibre B27 the end pool EGTA and CaCl_2 concentrations were 0.1 and 0.0082 mM; all other constituents of both pool solutions were as in Fig. 1. Insert trace *a* gives I_Q , with the calibration bar equal to 4 $\mu\text{A } \mu\text{F}^{-1}$ (current normalised to linear fibre capacitance). Insert trace *b* gives the best fit of equations (1) and (2) to a ΔA record minus the ΔA record (see Fig. 3A *a'*, *b'* and *c'*), with the calibration bar equal to $\Delta A_{720}/A_{550}$ of 1.0×10^{-2} . Each horizontal bar in (*a*) and (*b*) covers 1 ms and gives the average signal for that 1-ms interval. Trace (*a*) gives the signal averaged result of eight individual records and trace (*b*) is the average of two records. Both (*a*) and (*b*) were for a pulse to -20 mV in fibre B24 (see Fig. 2).

require that the rate coefficients for the calcium-troponin interaction be voltage-dependent (L.K., E.R. and M.F.S., in preparation). A more plausible alternative would be to introduce the troponin sites as a fourth compartment exchanging directly only with the myoplasm. The introduction of a fourth compartment would in general give rise to a third exponential term in the calcium transient. The absence of a third exponential term in ΔA_{ON} after s_{ON} thus requires that a possible fourth compartment exhibit special properties. A fourth compartment due to troponin might not give rise to a detectable deviation from a two exponential ΔA_{ON} : (1) if it became saturated with calcium early in the ΔA time course during s_{ON} ; (2) if it equilibrated rapidly with myoplasmic calcium and [Ca] were always well below levels for its saturation; (3) if it bound calcium only at a very slow rate; or (4) if it were relatively small. Possibility (1) seems unlikely considering that movement artefacts were observed only for the largest pulses and (4) seems unreasonable in view of the estimated concentration of about 70 μM for myoplasmic troponin molecules²² and the presence of four calcium binding sites per troponin molecule²³. Fast kinetic measurements of troponin calcium binding indicate that (2) might apply to the calcium-specific sites and (3) might apply to

the calcium-magnesium sites²⁴. Neither the rapidly nor slowly equilibrating sites would introduce deviations from a single exponential ΔA_{OFF} , providing the rapid sites were well below saturation and the slow sites were minimally filled during the preceding pulse.

Finally, a qualitatively different type of interpretation might be that during a pulse calcium equilibrates rapidly across the SR membrane and that the $[Ca]$ time course reflects the time and voltage dependence of the SR calcium permeability rather than

the time course of calcium redistribution under constant permeabilities. This type of interpretation seems rather unlikely because it would entail an energetically wasteful excess cycling of calcium between the SR and the myoplasm.

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Regulatory functions of the λ repressor reside in the amino-terminal domain

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The repressor of bacteriophage λ is a protein containing two domains of approximately equal size. Fragments containing the amino-terminal domain of repressor bind specifically to λ operator DNA and mediate positive and negative control of λ transcription both in vitro and in vivo.

THE bacteriophage λ repressor mediates positive and negative control of gene expression by binding to specific operator sequences in the phage DNA (see ref. 1 for review). Repressor is the product of the λ *cI* gene, and both the nucleotide sequence of *cI* and the amino acid sequence of repressor are known^{2,3}. The polypeptide chain of repressor (236 residues) is folded into two domains of roughly equal size connected by a protease-sensitive region of about 40 amino acids⁴. Cleavage of repressor with papain separates the two domains, and fragments containing each domain have been isolated and characterised⁴. The amino-terminal peptide, fragment *d*, consists of amino acids 1–92 of intact repressor. This fragment is monomeric and retains DNA non-specifically to nitrocellulose filters. In contrast, the carboxyl-terminal fragment (repressor residues 132–236) does not bind DNA but does dimerise. Dimerisation of repressor monomers is a prerequisite for strong binding of repressor to operator DNA⁵. We report here that the amino-terminal domain of repressor (fragment *d*) binds specifically to λ operator DNA and acts *in vitro* as a specific positive and negative regulator of transcription. Moreover, experiments with cloned fragments of the repressor gene *cI* demonstrate that the amino-terminal portion of repressor mediates these same regulatory functions *in vivo*.

The experiments reported in this article examine the interaction of the amino-terminal domain of repressor with the right operator (O_R) of λ DNA. As shown in Fig. 1, O_R contains three

17 base pair repressor binding sites which partially overlap two associated promoters P_R and P_{RM} . These promoters direct the divergent synthesis of *cro* and *cI* mRNA, respectively. The three repressor binding sites differ slightly in their nucleotide sequence^{6,7}, and repressor binds to these sites with an affinity order $O_{R1} > O_{R2} > O_{R3}$ ⁸. According to our current understanding, repressor bound to the strongest operator sites, O_{R1} and O_{R2} , prevents transcription from P_R and turns on transcription from P_{RM} (refs 1, 9 and B.J.M. and M.P., in preparation). At relatively high concentrations of repressor, the weakest operator site, O_{R3} , is also occupied and repressor synthesis from P_{RM} is shut off^{9,10}. Thus, the order of binding of repressor to the sites in O_R ensures that, at low concentrations of repressor, transcription of *cro* is turned off and transcription of *cI* is turned on, while at high repressor concentrations, transcription of both genes is turned off. λ Repressor also acts at another phage operator, O_L , to negatively control transcription initiated at the P_L promoter.

Residues 1–92 of λ repressor recognise operator DNA specifically

A protein bound to a DNA molecule can suppress or enhance the rate of chemical methylation by dimethylsulphate (DMS) of certain bases within its recognition sequence¹¹. Such DMS protection experiments have been used to demonstrate that a proteolytic fragment of *lac* repressor specifically binds to the *lac* operator¹², even though specific binding had not been detected in nitrocellulose filter binding experiments¹³. We report elsewhere that fragment *d* (amino acids 1–92 of intact λ repressor) retains DNA nonspecifically to nitrocellulose filters⁴. Here we use the more sensitive DMS protection technique to test whether fragment *d* can discriminate between operator and non-operator DNA.

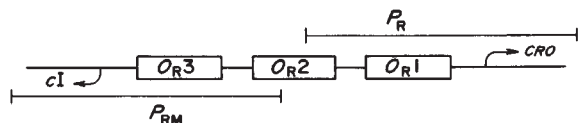


Fig. 1 λ O_R and associated promoters. The boxed regions represent 17-base pair recognition sites for λ repressor^{1,6}. The extent of the P_R and P_{RM} promoters has been defined by nuclease protection experiments¹. The transcriptional startpoints for *cI* and *cro* are shown.

In the experiment shown in Fig. 2a, fragment *d* and intact repressor were used to protect DNA containing the single operator site O_{R1} from methylation. The DNA was labelled, methylated either in the presence or absence of proteins, and cleaved so that the intensity of each band in the gel represents the extent of methylation of a single guanine in the upper strand of the operator site (see Fig. 3). The guanine residues are numbered according to the scheme shown in Fig. 3. Guanines on the lower strand of O_{R1} were examined in a separate experiment.

Fragment *d* (slot 1) and λ repressor (slot 2) have identical effects on the methylation of O_{R1} DNA, although higher concentrations of fragment *d* than repressor were needed to observe these effects. The methylation of G¹⁶ is enhanced whereas the methylation of G¹⁷, G¹⁹, G²⁰, and G²² is suppressed when compared with DNA methylated in the absence of either repressor or fragment *d* (slot 3). We also tested fragment *d* in methylation experiments in which G residues on the other strand of O_{R1} and on both strands of O_{R2} and O_{R3} were examined. In each case, the methylation pattern produced by fragment *d* at an operator site was identical to the pattern reported for intact λ repressor at the same operator site¹⁴.

The following control experiments confirm that the methylation protection pattern produced by preparations of fragment *d* results from specific binding of fragment *d* to operator DNA: (1) The protection observed with fragment *d* cannot be accounted for by supposing that it results from trace amounts of intact repressor in the fragment preparation. In Fig. 2b, methylation protection experiments were repeated using repressor (slot 5) and fragment *d* (slot 4) at the same concentrations as in Fig. 2a but higher concentrations of operator DNA were used. Fragment *d* still fully protects the operator DNA. The intact repressor does not because the operator DNA is present in excess. Since the concentration of repressor in slot 5 is 5% of the concentration of fragment *d* in slot 4 and yet fails to protect the operator DNA, even a 5% contamination of the fragment *d* preparation with intact repressor could not account for the observed protection. SDS-gel electrophoresis and amino acid composition data (ref. 4 and unpublished data) show that the fragment *d* used in these experiments cannot be contaminated with more than 2% intact repressor. Thus fragment *d* itself must be responsible for the observed methylation protection of λ operator DNA. (2) Repressor and fragment *d* protect only bases within the λ operator sites. Methylation of bases outside the operator (such as G²⁸ and G²⁹ in Fig. 2) is unaffected. Thus the pattern of methylation observed with repressor and fragment *d* is operator specific. (3) Other proteins do not protect bases within the λ operators, or produce different methylation protection patterns. The *lac* repressor, the basic protein lysozyme, and three carboxyl-terminal peptides of λ repressor (papain fragments *a*, *b*, and *c*)⁴ all fail to protect bases in the λ operators (data not shown). The λ *cro* protein binds to the λ operators but shows a slightly different pattern of methylation protection and enhancement¹⁵. Thus methylation patterns at the λ operators are specific for particular proteins and presumably reflect details of the interactions of these proteins with the DNA.

We conclude that fragment *d*, like intact λ repressor, binds specifically to λ operator DNA. Moreover, the methylation patterns suggest that repressor and fragment *d* make identical contacts with operator DNA.

From data as shown in Fig. 4, we can calculate an apparent equilibrium dissociation constant for the fragment *d*-O_{R1} interaction. Half-maximal protection of guanines on both the upper and lower strand of O_{R1} DNA occurs at a fragment *d* concentration of $3 \times 10^{-8} \text{ M} \pm 10^{-8} \text{ M}$. Thus the apparent dissociation constant for a complex between a fragment *d* monomer and the site O_{R1} is approximately $3 \times 10^{-8} \text{ M}$. (This assumes that our fragment *d* is 100% active with respect to operator binding.) At 0°C, this apparent equilibrium constant corresponds to a free energy change of $-9.3 \text{ kcal mol}^{-1}$ on binding of a fragment *d* monomer to O_{R1}. In similar conditions of temperature and ionic strength, the free energy change on binding of the intact repressor dimer to O_{R1} has been calculated to be $-16 \text{ kcal mol}^{-1}$ (R.T.S., in preparation). Thus, the interaction of fragment *d* with O_{R1} DNA is considerably weaker than that of the intact repressor dimer with the same operator site.

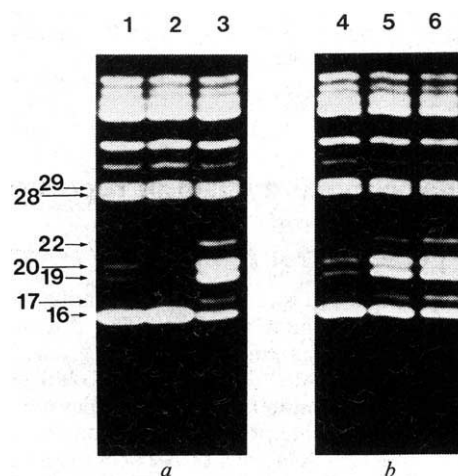


Fig. 2 Effects of λ repressor and fragment *d* on methylation of O_{R1} DNA. In slots 1–3, O_{R1} was present at a concentration of $4 \times 10^{-10} \text{ M}$ while in slots 4–6 its concentration was $2.8 \times 10^{-8} \text{ M}$. The methylation reactions were performed with the following additions: (1) $2 \times 10^{-7} \text{ M}$ fragment *d*; (2) $1.2 \times 10^{-8} \text{ M}$ λ repressor; (3) none (4) $2 \times 10^{-7} \text{ M}$ fragment *d*; (5) $1.2 \times 10^{-8} \text{ M}$ λ repressor; (6) none. Intact repressor and fragment *d* were purified as described elsewhere⁴. Their concentrations were determined by amino acid analysis and are given as monomer concentrations. In this experiment, O_{R1} was contained on the λ *Hind*II/*Hae*III 148 base pair restriction fragment labelled with ^{32}P at the 5'OH of the *Hind*II end. Labelled DNA was dissolved in 100 μl of 50 mM sodium cacodylate-HCl (pH 6.9), 2 mM CaCl_2 , 0.1 mM EDTA, and 100 $\mu\text{g ml}^{-1}$ bovine serum albumin. The DNA was pre-incubated with either repressor or fragment *d* for 30 min at 0°C. After this time, 1 μl of 99% dimethylsulphate (DMS) was added, and methylation was allowed to proceed for 30 min at 0°C. Destruction of unreacted DMS, EtOH precipitation, cleavage of the methylated DNA with piperidine, and electrophoresis on 20% acrylamide, 7M urea gels were carried out as described elsewhere^{11,24}. Autoradiography of the gel was with prefogged Kodak XR-5 film and Dupont intensifying screens at -80°C ²⁵.

The amino-terminal domain of repressor mediates positive and negative control of transcription *in vitro*

Meyer *et al.*⁹ have described the use of restriction fragments of λ DNA as templates for the synthesis of *cI* and *cro* transcripts directed by purified RNA polymerase. Purified λ repressor, added to this system at low concentrations, stimulates *cI* transcription and represses *cro* transcription. At higher concentrations, λ repressor turns off synthesis of both transcripts. The effects of fragment *d* on this *in vitro* transcription system are shown in Fig. 5. Increasing amounts of fragment *d* turn off synthesis of the *cro* transcript and stimulate synthesis of the *cI*

transcript. At higher concentrations of fragment *d* *cI* transcription is also turned off (data not shown). Thus, fragment *d*, like intact λ repressor, acts as a positive regulator of *cI* transcription and a negative regulator of both *cI* and *cro* transcription *in vitro*.

In these transcription experiments, the levels of *cro* turn-off and *cI* turn-on are similar to those observed with intact λ repressor⁹. As with the methylation protection experiments, however, greater concentrations of fragment *d* than repressor are necessary to observe the same levels of control.

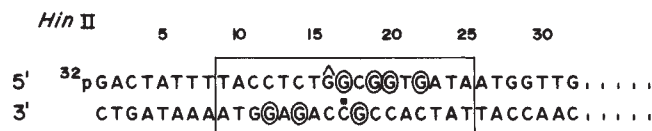


Fig. 3 Nucleotide sequence of λO_R1 and surrounding DNA. Bases within the boxed area have been identified as a λ repressor binding site¹. The dot at base 17 marks a pseudosymmetry axis in O_R1 . The λ repressor suppresses the methylation of those G residues that are circled and enhances the methylation of the G marked by a caret¹⁴.

The amino-terminal domain of repressor mediates positive and negative gene control *in vivo*

The plasmid pKB290 codes for the amino-terminal 118 amino acids—exactly half—of the λ repressor. The structure of this plasmid and details of its construction are given in Fig. 6. Transcription of the partial *cI* sequence on pKB290 is driven by a highly efficient *lac* promoter. The related plasmid pKB280, which contains the same *lac* promoter-*cI* fusion but carries the entire *cI* gene, directs the synthesis of 1–2% of a cell's protein as λ repressor¹⁶. Because the pKB290 construction generates an in-phase stop codon immediately following the codon for repressor amino acid 118, we expect this plasmid to direct the synthesis of substantial amounts of the amino-terminal half of λ repressor. As expected, cells carrying pKB290 contain a protein precipitated by anti- λ -repressor antibody, which on SDS gel

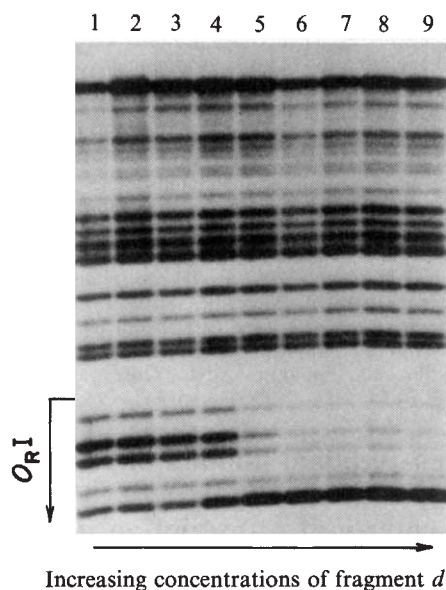


Fig. 4 Methylation of O_R1 DNA in the presence of increasing concentrations of fragment *d*. O_R1 (DNA 5×10^{-10} M) was methylated, cleaved, and analysed as described in Fig. 2. Each methylation reaction contained fragment *d* at the following concentration: (1) None; (2) 6.6×10^{-10} M; (3) 2×10^{-9} M; (4) 6.6×10^{-9} M; (5) 2×10^{-8} M; (6) 6.6×10^{-8} M; (7) 2×10^{-7} M; (8) 6.6×10^{-7} M; (9) 2×10^{-6} M.

electrophoresis migrates as predicted for a peptide of molecular weight 12,000–13,000 (data not shown). We do not know the precise intracellular concentration of this peptide, and so cannot exclude the possibility that its steady-state concentration may be lowered by proteolytic degradation.

In vivo the repressor fragment encoded by pKB290 binds to both λ operators, O_R and O_L , and turns off transcription from the corresponding promoters P_R and P_L . This is shown by the fact that cells carrying pKB290 are immune to phages λ , λV_2 , and $\lambda V_1 V_3$ but not to $\lambda V_2 V_1 V_3$. Phages λV_2 and $\lambda V_1 V_3$ bear mutations that reduce the affinity of repressor for sites in the left or in the right operator, respectively, while $\lambda V_2 V_1 V_3$ contains mutations in both operators. Cells containing pKB290 are sensitive to the heteroimmune phages λimm^{434} and λimm^{21} which bear operators different from those of phage λ . The detailed pattern of specific immunity of strains bearing pKB290 is the same as that of λ lysogens containing intact repressor.

The pKB290-encoded repressor fragment also mediates positive control of P_{RM} *in vivo*. This is shown by exploiting the properties of lysogens of a hybrid imm^{21} - λ phage. The construction and properties of this phage are detailed elsewhere^{1,10}. It carries the *lacZ* gene and synthesis of the β -galactosidase encoded therein depends on initiation of transcription at the λP_{RM} promoter. This hybrid phage produces no functional λ repressor and lysogens therefore produce very low levels of β -galactosidase since there is no repressor to stimulate P_{RM} . As shown in Table 1 and reported previously¹, if cells contain both the hybrid phage and a second prophage with a functional *cI* gene, then synthesis of β -galactosidase is stimulated. We report here that synthesis of β -galactosidase is also stimulated if lysogens of the fusion phage contain pKB290. Thus the pKB290-encoded repressor peptide, like intact repressor, turns on P_{RM} -initiated transcription *in vivo*.

Discussion

Recent results from this laboratory demonstrate that λ repressor consists of two stably folded domains of similar size, connected by a protease-sensitive region of about 40 amino acids⁴. We have shown here, using dimethylsulphate protection experiments, that a fragment containing the amino-terminal domain binds specifically to the λ operators, and in fact seems to make all the same contacts with operator DNA as does whole repressor. Thus the amino-terminal domain of repressor recognises operator DNA, while the carboxyl-terminal domain, as we showed previously, contains contacts important for repressor

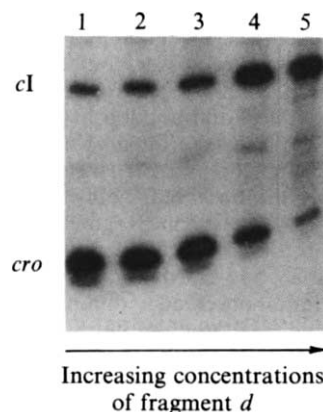


Fig. 5 Effects of fragment *d* on transcription *in vitro*. The *cro* and *cI* RNAs were transcribed from the λ HaeIII '790' base pair restriction fragment as described⁹. Transcription reactions contained the following concentrations of fragment *d*: (1) None; (2) 9×10^{-7} M; (3) 1.8×10^{-6} M; (4) 4.5×10^{-6} M; (5) 9×10^{-6} M; (6) 1.8×10^{-5} M. After addition of fragment *d*, RNA polymerase (4×10^{-8} M) was added in fourfold excess (mol/mol) over template DNA. In this experiment, maximum stimulation of *cI* transcription was fivefold. This is comparable to the maximum stimulation observed with intact λ repressor in this system (data not shown).

Table 1 P_{RM} -directed synthesis of β -galactosidase

Lysogen	β -galactosidase
RV308(λ 112 <i>cIsus</i> 34)	125
RV308(λ 112 <i>cIsus</i> 34; λh^{80})	1,200
RV308(λ 112 <i>cIsus</i> 34)/pKB290	850

See refs 1 and 10 for methods and strain construction.

dimerisation. Similar conclusions about the function of the amino- and carboxyl-terminal regions of repressor have been previously reached based on genetic studies of mutant repressors¹⁷⁻²⁰. We do not know which of the amino-terminal 92 residues of repressor contact the operator. However, the amino-terminal 40 amino acids contain many polar and charged side chains which afford ample possibilities for hydrogen bond formation with operator DNA²¹. This region contains 10 lysine and arginine residues, and it has been suggested that some of these basic amino acids may form ionic contacts with the negatively charged phosphates of the operator DNA backbone^{3,21}.

Although the *lac* repressor shows no amino acid sequence homology with λ repressor, the two molecules are similar in several ways. A proteolytic fragment containing the amino-terminal 59 residues of *lac* repressor specifically binds to *lac* operator DNA^{12,13}, while a fragment containing about 300 carboxyl-terminal amino acids mediates formation of stable tetramers of *lac* repressor²². Thus both *lac* and λ repressor contain two domains, albeit of different sizes, and the respective amino- and carboxyl-terminal domains of these repressors mediate similar functions of operator DNA recognition and oligomerisation.

Not only does the amino-terminal domain of λ repressor specifically recognise λ operator DNA, but our results indicate that it can mediate both positive and negative control of gene function. Sufficiently high concentrations of repressor fragments containing the amino-terminal domain mimic the regulatory actions of intact repressor both *in vitro* and *in vivo*. As mentioned previously, the pattern of positive and negative gene control by repressor depends on the order in which specific operator sites are occupied. Thus, the order of binding of repressor's amino-terminal domain to the operator sites must be similar to that of intact repressor. Furthermore, we conclude that the carboxyl-terminal 144 amino acids of repressor cannot be essential for negative control (which apparently occurs by steric exclusion of RNA polymerase from its promoters) nor for positive control. One model for positive control is that repressor provides a protein-protein contact that stabilises RNA polymerase binding at the P_{RM} promoter. While our results do not exclude such a model, they do eliminate the possibility that the carboxyl-terminal region of repressor is responsible for such a contact.

Recent results (R.T.S., in preparation) support the earlier conclusion that dimers of λ repressor are the form which bind strongly ($K_D = 3 \times 10^{-13}$ M) to the λ operators⁵. What part do the two amino-terminal domains in an intact repressor dimer play in operator DNA binding? The fact that the λ operator sites have an axis of partial two-fold rotational symmetry through the ninth of their seventeen base pairs⁶ suggests that in each dimer-operator complex the two amino-terminal domains contact symmetrically related sets of base pairs. The following observations are consistent with the suggestion that both amino-terminal domains contact the operator when the repressor dimer binds: (1) Monomeric fragment *d* makes the same contacts with the operator as does repressor, but its affinity for the operator is substantially less than that of the intact repressor dimer. This would be expected if both amino-terminal domains of the repressor dimer contact the operator and contribute to the total DNA binding energy. (2) Fragment *d* is a relatively compact molecule⁴. Gel filtration experiments suggest that its longest dimension is less than 37 Å (R.T.S. and C.O.P., unpublished). Intact λ repressor specifically contacts a single operator site (such as O_R1) at positions spanning 58 Å, and protects from

nuclease digestion a sequence of 21 bases centred on the partial two-fold symmetry axis of the operator site (A. Johnson, unpublished). At sufficiently high concentrations, fragment *d* protects precisely the same bases from nuclease as does intact repressor (A. Johnson, unpublished). In the absence of an extraordinary conformational change, a single fragment *d* molecule could not make all the same contacts as the intact repressor dimer. Thus, we assume that two fragment *d* molecules bind to a single operator site.

The above model explicitly predicts that there should be two fragment *d* binding sites in a single 17-base pair operator site. The size of fragment *d* suggests that it could interact with at most 10–11 base pairs in B-form DNA, and thus the nine base pair pseudosymmetric 'half-sites' would seem to be likely candidates for fragment *d* recognition sequences. We have found that half-maximal protection or enhancement of guanines in both 'half-sites' of O_R1 occurs at an identical concentration of fragment *d*. This could indicate either that there are two independent sites with roughly equal affinities for fragment, or that binding of the fragment to both sites is coupled or cooperative, perhaps as a consequence of energetically favourable protein-protein contacts made while the fragments are bound to the operator site. The possibility of such contacts is not excluded by our finding that fragment *d* is a monomer⁴. That conclusion was based on experiments performed at fragment concentrations (5.5×10^{-5} M) where interactions involving a free energy change of only a few kcal per mol would not have been detected.

When λ lysogens are treated with inducing agents such as mitomycin C or UV light, the repressor is cleaved and the prophage induced²³. This cleavage occurs between repressor residues 111 and 112 thereby separating the amino- from the carboxyl-terminal domains (M. Ross, R.T.S., and M.P., in preparation). The results reported in this article suggest that the induction cleavage does not destroy the DNA binding site of repressor. Rather, we presume that the cleaved, amino-terminal

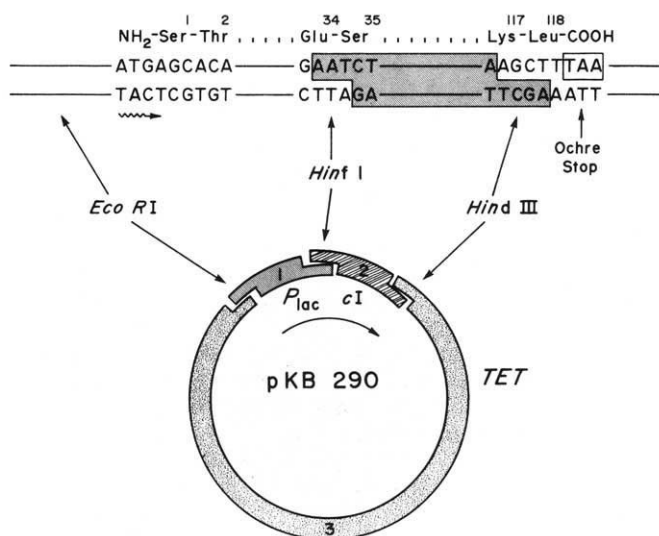


Fig. 6 Structure of pKB290. pKB290 was constructed by *in vitro* ligation of three pieces of DNA (see refs 16 and 26 for methods). The partial *cI* gene of pKB290 was reconstructed from the *EcoRI*-*HinfI* restriction fragment (containing P_{lac} and the amino-terminal portion of *cI*) isolated from pKB280¹⁶ and the *HinfI*-*HindIII* restriction fragment (containing sense bases 104–352 of *clind*) isolated from the *ind*⁻ analogue of pKB252²⁶. These two fragments were cloned between the *EcoRI* and *HindIII* sites of pMB9²⁷, and tetracycline resistant clones of *Escherichia coli* 294 were selected. About 50% of the tetracycline resistant clones were immune to phage λ infection. Typical of these was pKB290. Restriction analysis of pKB290 DNA confirms the structure shown above. The DNA and protein sequences shown are those predicted from the known sequence of λ *cI*² and pMB9²⁸. Note that the pKB290 *cI* peptide contains lys¹¹⁷ like *ind*⁻ λ repressor.

induction fragment cannot dimerise and thus that its affinity for the operators is reduced. It is this reduction in operator affinity that results in derepression and thus induction. However, the immunity properties of cells carrying plasmid pKB290 show that high concentrations of fragments bearing the amino-terminal domain of repressor can maintain repression of the λ operators. This suggests that prophage induction would not occur in cells containing large amounts of wild-type repressor, even if that

repressor were efficiently cleaved.

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letters

Detection of Jupiter's ring at 2.2 μ m

FOLLOWING the announcement of the discovery of a ring around Jupiter by the Voyager spacecraft¹ a successful attempt was made to detect the reflected sunlight from this ring at 2.2 μ m. We describe here the results of this experiment and the precautions taken to separate the radiation from the ring and the scattered radiation from the planet.

The observations were made on 10 and 11 March 1979 UT using the 2.2-m University of Hawaii telescope on Mauna Kea, an *f*/35 chopping secondary photometric system and an indium antimonide photovoltaic detector. Most of the measurements were made with a 5 arc s diaphragm centred $\sim$ 13 arc s above the limb of the planet. The outer edge of this diaphragm corresponds to the maximum radius of the ring as reported from the Voyager experiment. At the time of the observations the planet had a diameter of 41 arc s and was at a distance of 4.6 AU from the Earth. The jovian equator was aligned at an angle of 14° to the Earth's Equator. The shaded circles in Fig. 1 show where most of the observations were made. There is considerably stronger emission coming from the positions in the jovian equatorial plane than from the positions at higher jovian latitudes. We attribute this excess to sunlight reflected from the ring of Jupiter. The net flux density from the ring through a 5 arc s diaphragm on either side of the planet is 1.5 ± 0.3 mJy corresponding to a 2.2- μ m magnitude of 14.0. The emission seems to be symmetrical about the centre of the planet.

The main problem in measuring the ring of Jupiter is the elimination of stray radiation from the planetary disk that is scattered by the Earth's atmosphere, the telescope and the photometer. The following procedures were therefore used to separate the ring from the background. (1) The search for the ring was carried out at 2.2 μ m, using a standard 0.4- μ m wide photometric band. At this wavelength the strong molecular absorption feature in Jupiter's atmosphere² reduces the scattered radiation by a factor of about 30 below that of adjacent shorter wavelengths. (2) Observations were made using a small

chopper spacing (8 arc s) at the four shaded positions shown in Fig. 1. The flux densities shown on Fig. 1 represent the difference between the flux densities from the shaded circles and the mean of the flux densities from the open circles immediately north and south of them. (3) Measurements were made both at the expected positions of the ring, on each side of the planet, and at the symmetrical positions north and south of an east–west line through the planet's centre. As the secondary support system on the 2.2-m telescope is symmetrical about both the north–south and the east–west axes as the chopping direction is directly along a north–south line, and as the 2.2- μ m surface brightness from Jupiter is quite uniform there should be no significant difference between the scattered emission from the four shaded regions in Fig. 1. Observations at 1.65 μ m showed this to be true.

As checks on the symmetry of the scattered light from the planet the following tests were also made: (4) each observation at 2.2 μ m was sandwiched between two measurements at 1.65 μ m. At the latter wavelength, which is well outside the absorption bands, scattered radiation from the planet dominates that reflected from the ring. The average 1.65 μ m emission from the shaded areas was the same along a north–south line to within a factor of 1.1, although a somewhat greater east–west asymmetry was indicated. (5) The IR photometer was rotated 90° and the observations repeated to check the possibility that asymmetries in the photometer are the cause of the differences in 2.2- μ m flux density in Fig. 1. No significant variation was found. (6) 2.2- μ m observations were made of the scattered radiation at distances of 15 and 25 arc s from a bright star. No north–south asymmetries above 15% in the scattered radiation were detected.

The final results shown in Fig. 1 are the means of five observations each of 4 min integration time. These observations were timed to avoid Amalthea and the Galilean satellites. The main source of error is fluctuation in the scattered background due to imperfect tracking of the telescope during the observations. Statistical errors were several times smaller.

Limited observations were made to determine the spatial distribution of the emission from the ring along the equatorial

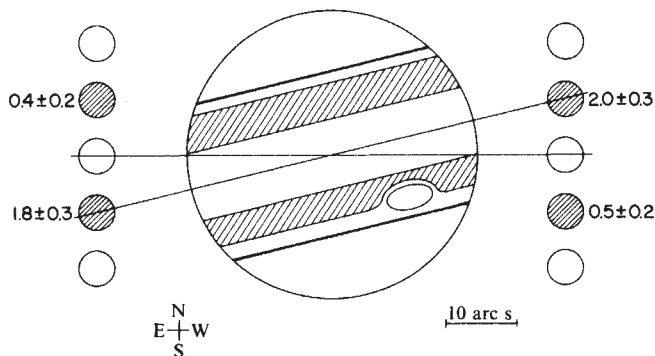


Fig. 1 The positions at which there was a search for 2.2- μ m emission. The numbers are the differences between the flux densities (mJy) from the shaded circles and the mean of the flux densities from the open circles immediately to the north and south.

line of the planet. These measurements showed no detectable emission at distances > 17 arc s above the limb, and no increase in emission at distances closer to the planet. One measurement at 13 arc s above the limb with a 7.5 arc s diaphragm gave a flux density attributable to the ring a factor of 1.5 ± 0.4 greater than that in a 5 arc s diaphragm.

A measurement was made of the 2.2- μ m flux density of the rings of Saturn on the same night as the Jupiter observations. The flux density through a 5 arc s diaphragm at the tip of Saturn's rings was 22 Jy. The ring of Jupiter is therefore about 10.4 mag fainter than those of Saturn as measured through the same diaphragm. If both ring systems are comprised of similar particles, and we were viewing approximately the same fraction of each system, then the number of particles in Jupiter's ring is of the order of 10^5 less than that in Saturn's.

An unsuccessful search was made for thermal emission from Jupiter's ring at 20 μ m. A 3σ upper limit of 1.5 Jy was established for the flux density in a 6 arc s diaphragm 13 arc s above the surface.

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γ Rays from close binaries in the quiet stage

THE presence of rotating neutron stars in binary systems is revealed by the existence of X-ray pulsators which are generally associated with massive companions. The evolutionary history of these systems is now relatively clear (see ref. 1 and refs therein). The X-ray phase, which corresponds to accretion on the neutron star of a strong stellar wind $\dot{M} \approx 10^{-6} M_{\odot} \text{ yr}^{-1}$, is preceded by a much longer quiet state, where the primary is unevolved, possibly with a weak wind, $\dot{M} \approx 10^{-9} M_{\odot} \text{ yr}^{-1}$ and the rotational energy loss of the neutron star inhibits accretion.

However, with the only exception of PSR1913+16, radio pulsars are not found in binary systems. Here, motivated by the recent discovery of γ -ray emission from slow pulsars^{2,3}, we suggest γ -ray observations as a way of detecting binaries in the quiet state and compare the expected number with the COS B results. We refer to the review of van den Heuvel¹ where, as a representative case, a system of initial mass $20+8 M_{\odot}$ and orbital period 4.7 d is considered. After the first stage of mass exchange and the supernova explosion, one has a neutron star with an unevolved companion of $22.7 M_{\odot}$. The period is now $P = 12.6$ d. The primary remains on the main sequence (quiet stage) for $t_{\text{ms}} = 3.6 \times 10^6$ yr. The X-ray phase occurs after the star has left the main sequence, but before it fills its Roche lobe, as an excessive mass transfer absorbs the X-ray emission. Its duration is $t_x \approx 10^4$ yr.

During the quiet stage the pulsar emission interacts with the weak wind of the main sequence primary. At first, when the pulsar luminosity is still large, the pulsar emission mechanism is unaffected by the wind, although the radio signal can be absorbed. The free free opacity at radio wavelengths is given by⁴

$$\tau_{\text{ff}} = 2 \times 10^{-23} n^2 T^{-3/2} \lambda^2 A$$

where n is the density, T the temperature, λ the wavelength and A the separation of the system.

Taking $T = 10^5 T_{\odot}$ K, $\lambda = 100 \lambda_{100}$ cm, and $A = 10^{12} A_{12}$ cm one has $\tau_{\text{ff}} \approx 1$ for

$$n \geq 1.3 \times 10^7 T_{\odot}^{3/4} \lambda_{100}^{-1} A_{12}^{-1/2} \text{ cm}^{-3}$$

corresponding to a mass loss

$$\dot{M} > 4\pi A^2 \rho V = 3.6 \times 10^{-10} M_{\odot} \text{ yr}^{-1}$$

where ρ is the density and the wind velocity V is taken $V = 10^8 \text{ cm s}^{-1}$. Since the derived mass loss is typical of a massive main sequence star, it is not surprising that no radio pulsar has been detected in close massive binaries. This stage ends when the accretion process sets in destroying completely the pulsar emission mechanism. The condition that no accretion occurs is⁴

$$\rho V^2 < L/4\pi r_A^2 c$$

where L is the total power of the pulsar, and $r_A \approx GM/V^2$ the capture radius. Taking $M = 1 M_{\odot}$, $n = 2 \times 10^7 \text{ cm}^{-3}$, and the same wind parameters derived above, then

$$L > 1.4 \times 10^{31} \text{ erg s}^{-1}$$

This luminosity corresponds to a pulsar of $P \approx 1$ s and age between 10^6 and 10^7 yr, which indicates that the pulsar luminosity is sufficient to inhibit accretion from a weak wind for a time comparable to t_{ms} . For $P < 1$ s the light cylinder is within r_A and the plasma is confined to $r > r_A$.

The observation^{2,3} that three isolated pulsars with age $\approx 10^5$ yr emit γ rays with very high efficiency, and $L_{\gamma} \approx 10^{33} \text{ erg s}^{-1}$, suggests that the systems considered above could be γ -ray emitters for a consistent fraction of t_{ms} . In fact the wind would be transparent to γ rays. Together with γ rays, the pulsar is expected to produce relativistic particles which, interacting with the surrounding plasma, could enhance the γ ray yield and produce high frequency radio emission which would not suffer absorption. X rays could also be produced at the shock discontinuity between the relativistic pulsar wind and the stellar wind. The X-ray power should be at most comparable to the γ -ray power, since the latter accounts for nearly all the pulsar rotational energy loss. Note that a picture of this kind was proposed to explain the X and γ -ray emission of Cyg X-3 (refs 5, 6). In that case, however, the binary is of low mass, with $P = 4.8$ h and $A = 10^{11}$ cm and the pulsar should be extremely young, 10^3 – 10^4 yr.

It is estimated¹ that there are some 2,400 systems in the quiet stage in the Galaxy and some 120 within 3 kpc. These numbers are deduced starting from the number of close binary systems in the Galaxy, with $M_1 > 15 M_{\odot}$ ($\approx 20,000$). The observability of the γ -ray emission from these systems depends on the intrinsic γ -ray luminosity and on the beaming. Instead of estimating

these highly uncertain parameters, we normalise to the total number of isolated pulsars of the same age. Taking for isolated pulsars the birth rate function given by Gailly *et al.*⁷ $= (1.3-11) \times 10^{-5} \text{ kpc}^{-2} \text{ yr}^{-1}$ one has that within 3 kpc there are $(3.5-30) \times 100$ pulsars of age $3 \times 10^6 \text{ yr}$. Therefore pulsars in binaries seem to be 30–3% of the isolated ones.

The total number of pulsars already observed in γ rays is 5, and a large fraction of the 20 unidentified γ -ray sources⁸ may be pulsars as well. The estimated percentage of pulsars in binaries then indicates that among these sources there could be one or more γ -ray emitting pulsars in binary systems.

The pulsed radio emission would probably be absent, making the identification arduous, because the low fluxes make it impossible to detect a short period modulation in γ rays without knowing the period *a priori*.

The X-ray flux would be lower than the γ -ray one, even if the luminosities are comparable, because the X-rays are not beamed, but could be detectable with a high sensitivity instrument. The high-frequency radio emission could also be observable. In both cases the search for an optical identification, impossible in the large γ -ray error box, could be fruitful. In our picture the counterpart would be a massive main sequence star (B0–B4). For these systems eclipses are probable and they could be observable in γ rays, thus confirming the identification. If the mass function also were known, the present picture could hopefully be distinguished from that of an accreting black hole⁹ or other systems.

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Negative wake behind bubbles in non-newtonian liquids

GAS bubbles rising by gravity in non-newtonian elastic liquids^{1–4} are different to gas bubbles in viscous newtonian fluids in at least two ways. First, the bubbles in the non-newtonian liquids often have a peculiar tip at the rear pole, and second, the terminal rise velocity versus volume curve often has a discontinuity at a certain 'critical' volume. To investigate this unusual flow situation further we have used laser-Doppler anemometry⁵ to measure the liquid velocity in the wake behind air bubbles in a non-newtonian liquid. The measurements described here reveal the unexpected result that the liquid velocity behind the bubbles is in the downwards direction away from the rising bubbles (the velocities are referred to an observer at rest with respect to the liquid far from the bubbles). Thus the liquid velocity is opposite to the velocity in the usual wake behind objects moving in viscous newtonian fluids and we have called the phenomenon 'negative wake'.

The measurements were made on gas bubbles rising along the axis of a vertical glass cylinder. Consider first in Fig. 1 the fluid velocity on the axis of a gas bubble rising in a viscous newtonian

liquid (a 99% glycerol–1% water mixture at 295 K). The position and velocity of the bubble is indicated in Fig. 1 by the width and height of the rectangle. Figure 1 shows that while some liquid is pushed ahead in front of the bubble more liquid is pulled along behind the bubble, thereby constituting the 'wake' of the bubble. A knowledge of the flow field in the wake behind bubbles in viscous newtonian fluids has helped in studies of bubble coalescence^{6–7}, where the wake behind a leading bubble is responsible for the increased speed with which a trailing bubble catches up with the leading bubble so that coalescence can take place.

We can now consider the non-newtonian liquid, a 1% solution of polyacrylamide in glycerol. This is a highly elastic liquid with a zero-shear-rate viscosity^{8–9} of about 52 Pa s^{-1} at 295 K. The shape of a bubble rising in this fluid is shown in Fig. 2. These photographs are of the same bubble but taken in two mutually

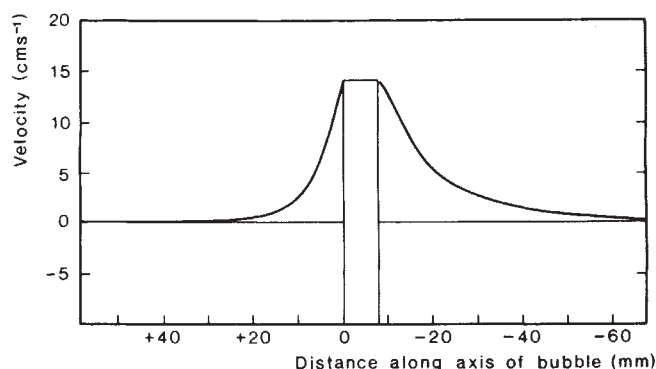


Fig. 1 Axial component of liquid velocity on the axis of an air bubble of volume 400 mm^3 rising in a viscous newtonian liquid (a 99% glycerol–1% water mixture at 295 K). The position of the bubble is indicated by the tall rectangle, the height of which indicates the rise velocity of the bubble (14.2 cm s^{-1}). The data are obtained from an experiment in which a bubble rising at constant speed along the axis of a glass cylinder passes a test section in which two photocells measure the rise velocity of the bubble and a laser-Doppler anemometer measures the axial component of the liquid velocity at a fixed point on the axis of the cylinder as a function of time. A third photocell connected to one of the anemometer beams indicates exactly when the bubble enters into and exits from the point of the velocity measurement. The constant rise velocity of the bubble allows us to transform the velocity versus time record into the velocity versus space representation shown.

perpendicular directions. The photographs reveal that the shape of the bubble at the rear pole is not that of a cusp but that of a knife edge. The knife edge was exhibited by all bubbles with a volume larger than about 50 mm^3 . Bubbles in the volume range $20-50 \text{ mm}^3$ showed a weak cusp, and bubbles smaller than about 20 mm^3 had a perfectly rounded rear pole.

Figure 3 shows the velocity field on the axis of a bubble of the same size as that shown in Fig. 2. Comparison with Fig. 1 clearly shows that the fluid behind the bubble in the non-newtonian liquid instead of being pulled along in the usual wake pattern is in fact 'recoiling' away from the bubble. Adding particles to the fluid reveals this negative wake to the naked eye.

The magnitude of the negative wake depends on the size of the bubbles and for bubbles larger than 460 mm^3 is even more pronounced than in Fig. 3. The smallest bubble for which a velocity signal could be obtained had a volume of 250 mm^3 and this also showed a small negative wake. Thus the negative wake has been established only for bubbles with a knife-edge rear,

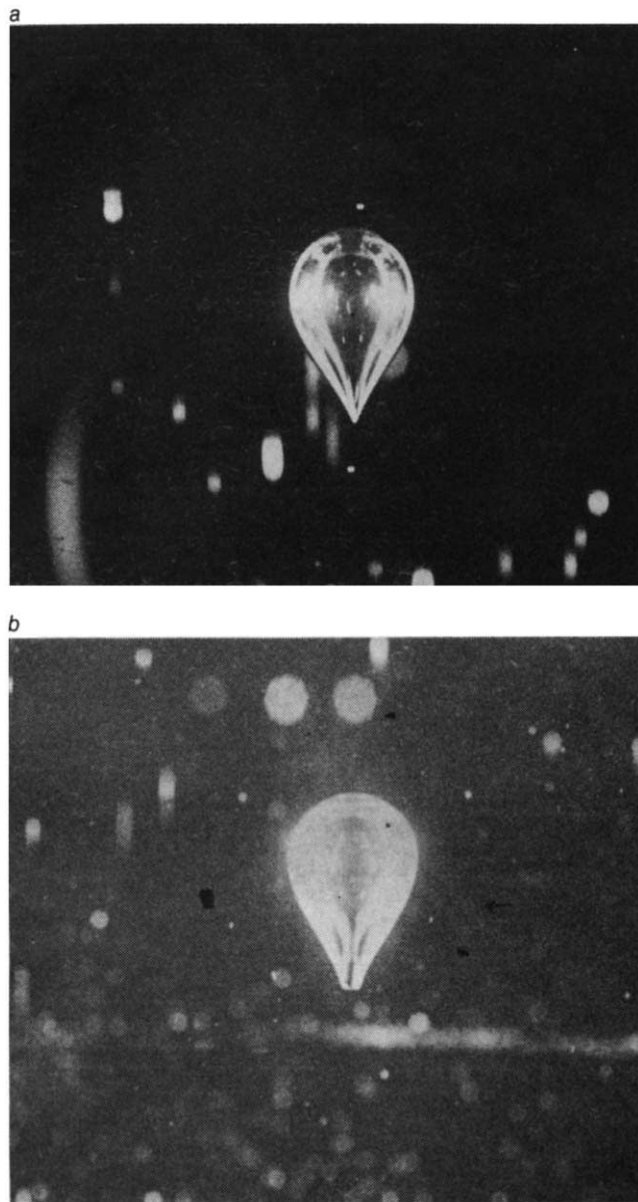


Fig. 2 *a*, An air bubble of volume 460 mm^3 rising in a solution of polyacrylamide in glycerol at 295 K. *b*, As in *a* but with the photograph taken in a direction perpendicular to that of *a*.

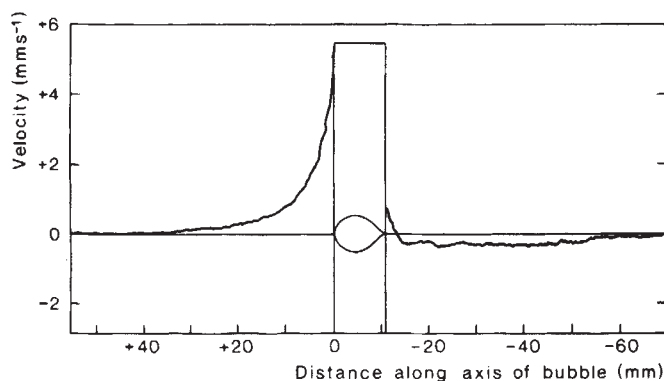


Fig. 3 Representation similar to Fig. 1 of the axial component of liquid velocity on the axis of an air bubble of volume and shape as shown in Fig. 2 rising in a solution of polyacrylamide in glycerol.

around which the entire flow field (particularly in the neighbourhood of the rear pole) will not be rotationally symmetric. This negative wake is just one among several⁹ phenomena where the elastic properties of a fluid give rise to an effect that is opposite to that which would be caused by inertial forces.

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Bubbles in a freshwater lake

WHEN the wind is strong enough to produce whitecaps on Loch Ness, patchy 'clouds' of acoustic reflectors are detected well below the surface, the depth to which they penetrate increasing with wind speed (Fig. 1). No seasonal variation in the occurrence of the reflectors has been detected. A biological explanation is therefore discounted and we suggest here that they are bubbles caused by waves breaking and forming whitecaps in deep water. Similar bubble clouds may occur in other lakes and in the sea.

The instrument used was a 248 kHz inverted echo sounder moored at 79m below the surface some 250m off-shore where the water was 166 m deep. The beam diameter was such that a trawl float target towed across the beam near the surface remained 'visible' for about 7 m.

The base of the bubble cloud had a variable structure, sometimes appearing as a series of 'fingers' penetrating downwards into the water column (see Fig. 1*m*) and sometimes having a sheared appearance (see Fig. 1*e*) reminiscent of billows seen on the tops of cumulus clouds in strong winds. Both these short-period fluctuations of a minute or so and longer period fluctuations (see Fig. 1*j*) of 5–10 min were observed.

The bubbles penetrate surprisingly far into the water column (Fig. 2), the mean depth d (cm) being approximately related to the wind speed 10 m above the surface, W_{10} (cm s^{-1}), by

$$d = 0.4(W_{10} - 250), \quad 0 < W_{10} < 1,400 \quad (1)$$

A simple model has been constructed to interpret this observation. The bubbles are supposed to be carried down by a steady vertical component, w , of current. Being buoyant, however, their actual descent rate is approximately given by

$$\frac{dp}{dt} = g\rho \left(w - \frac{ar^2g}{\nu} \right) \quad (2)$$

where $p = p_0 + g\rho d$ is the pressure, p_0 is atmospheric pressure, g acceleration due to gravity, ρ the density of water, ν the molecular viscosity, and d the depth of a bubble of radius r at time t . The coefficient a is $1/3$ for 'clean' bubbles of sufficiently small Reynolds number, but approximately $2/9$ for 'dirty' bubbles covered by a surface contaminant¹. Bubbles of radius greater than $(w\nu/ga)^{1/2}$ will not be carried down.

The bubble volume obeys the gas law

$$\frac{4}{3}\pi r^3 = nRT/(p + 2\Gamma/r) \quad (3)$$

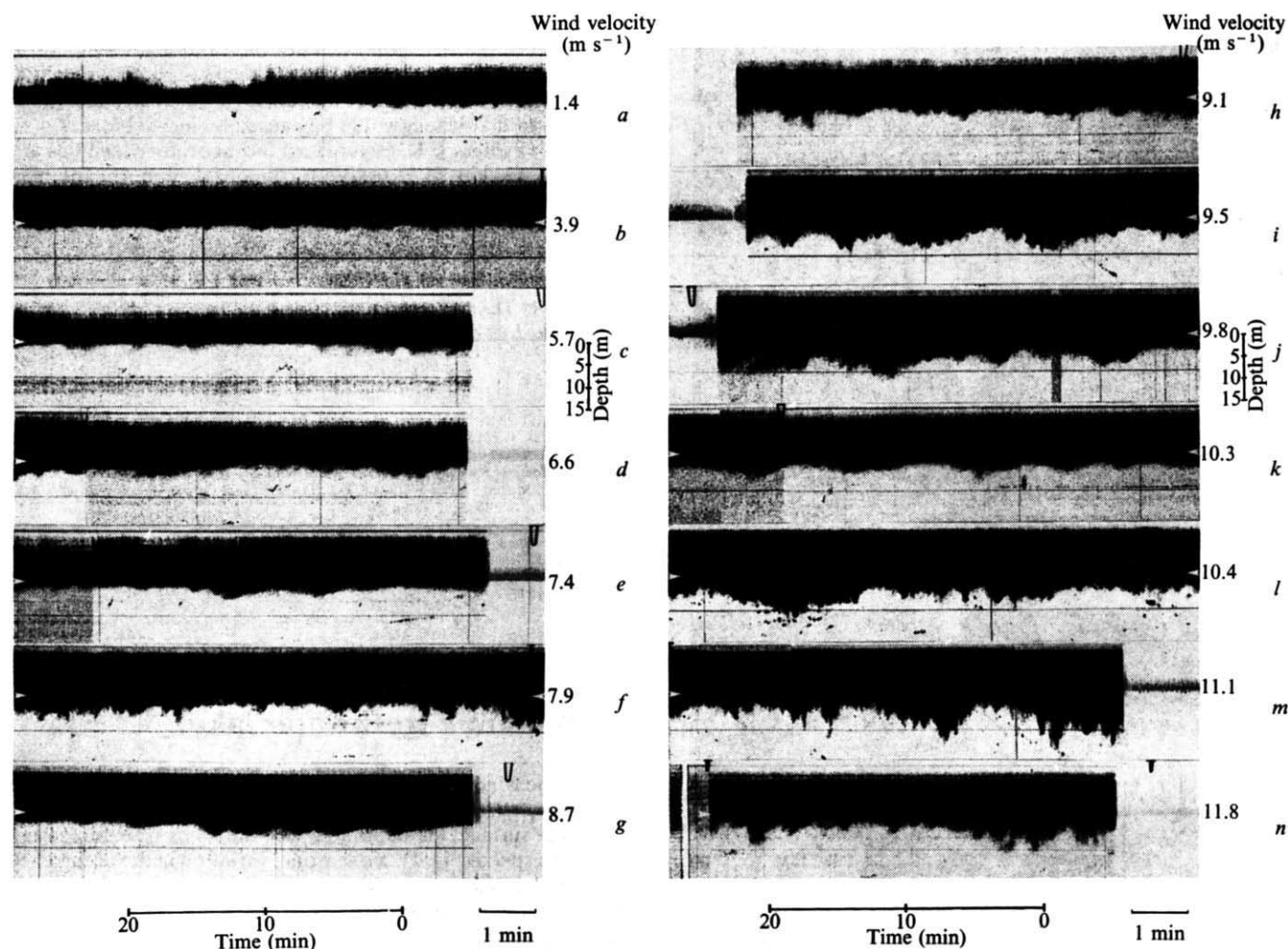


Fig. 1 Clouds of acoustic bubble reflectors below the surface of Loch Ness. The observations were made ~250 m off-shore where the fetch exceeds 11 km for north-easterly or south-westerly winds. The wind speed corrected to 10 m is shown. Depth increases downwards and time to the left. The position of the surface is marked by arrows or by a short sequence (such as at the left of *e*) where the gain of the sounder has been reduced and the recording speed increased so that surface waves may be seen. The acoustic echoes are the dark regions. Occasional individual echoes are probably fish, although in (*l*), from November, the individual echoes below the bubble cloud at the right may be dead leaves with air caught in their membrane structure. Similar echoes were seen when leaves were deliberately distributed over the sounder. Echoes from above the surface level derive from multiple scattering at the surface, as well as from the finite pulse length, 0.2 or 0.5 ms, of the sounder. The horizontal and vertical lines are reference lines on the recorder.

where n is the number of moles of gas in the bubble, R is the gas constant, T the temperature and Γ the surface tension. Gas is lost from bubbles by passing into solution. We take the diffusion equation^{2,8}

$$\frac{dn}{dt} = -4\pi br^2 \left(p + \frac{2\Gamma}{r} - p_0 \right) \quad (4)$$

where

$$b = \begin{cases} \frac{2}{3\pi} \left(\frac{\pi g D}{2\nu} \right)^{1/2} \frac{r^{1/2} K}{M} & \text{for clean bubbles,} \\ \frac{2}{\pi} \left(\frac{2g D^2}{9\nu} \right)^{1/3} \frac{K}{M} & \text{for dirty bubbles,} \end{cases}$$

and D is the diffusivity of the bubble gas in water, M is its molecular weight and K is its coefficient of absorption. The forms of b are applicable at high bubble Peclet number. We assume that the water is saturated with respect to the bubble gas at atmospheric pressure.

If T is constant (a good assumption as temperature varies little in the mixing layer) we obtain from equations (3) and (4)

$$\frac{dr}{dt} = - \left[3b \left(p + \frac{2\Gamma}{r} - p_0 \right) / 4\pi + r \frac{dp}{dt} \right] / \left(3p + \frac{4\Gamma}{r} \right) \quad (5)$$

The increase of radius due to bubble coalescence, potentially an important factor in freshwater where coalescence is more rapid than in seawater³, is ignored on the assumption that the probability of bubble encounter may be small other than perhaps in the injection zone near a breaking whitecap.

The sonar will detect only bubbles of radius about equal to, or exceeding, a 'resonant' radius

$$r = \frac{1}{2\pi f} \left(\frac{3\gamma p}{\rho} \right)^{1/2} \quad (6)$$

where f is the sonar frequency and γ is the ratio of specific heats of the bubble gas. This radius is about 0.0015 cm at atmospheric pressure. Equations (5) and (2) have been numerically integrated to find the depth at which a bubble of given radius at the surface will 'vanish' from the sonar, when carried down in current w . The greatest depth to which bubbles can be carried is shown as a function of w , in Fig. 3. For clean bubbles (w , cm s⁻¹) this maximum depth is

$$d \sim 52.6w \quad (0 < d(\text{cm}) < 310, 0.0015 < r(\text{cm}) < 0.013)$$

whereas for 'dirty' bubbles

$$d \sim 92.1w \quad (0 < d(\text{cm}) < 400, 0.0015 < r(\text{cm}) < 0.014)$$

Taken with equation (1) these imply relations between the

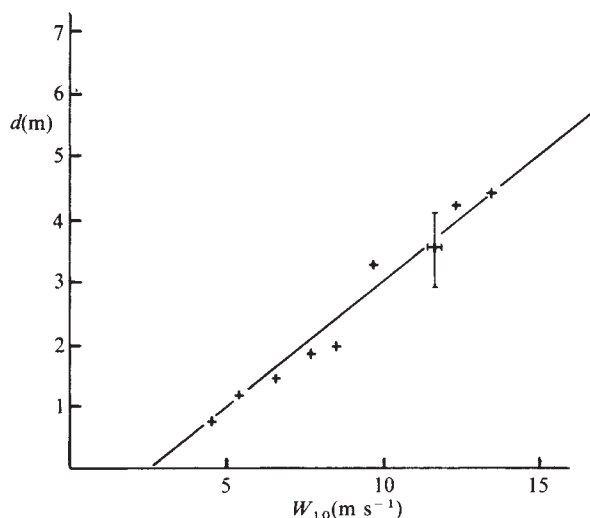


Fig. 2 The average depth, d , of the observed cloud of reflectors as a function of wind speed, W_{10} . Each point is an average of several estimates based on records of over 30 min. The standard deviation of one point is shown.

vertical velocities of the turbulent motion, w (cm s^{-1}) and the wind W_{10} (cm s^{-1});

$$w \sim 0.0076(W_{10} - 250) \quad (\text{clean bubbles})$$

and

$$w \sim 0.0043(W_{10} - 250) \quad (\text{dirty bubbles}). \quad (7)$$

Although whitecapping is reduced by surface contaminants, it is unlikely that the bubbles are clean and the estimate of vertical speeds for dirty bubbles seems more appropriate. The estimate of vertical currents in equation (7) is large but, especially in view of the assumptions, not unreasonable if one accepts an estimate of the surface current, $u \sim 0.03 W_{10}$, and as $(w^2)^{1/2} \sim 0.066u$ in a turbulent boundary layer⁴. The model predicts that bubbles 'vanish' within a few minutes.

Individual clouds may penetrate to more than twice the depth given by equation (1). This is much greater than the wave height. It therefore seems that bubbles, though caused by breaking waves, must be carried downwards by other larger scale processes, and Langmuir circulation seems a possible contributor⁵.

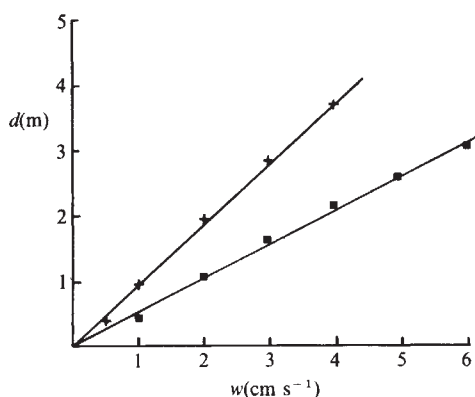


Fig. 3 The maximum depth, d , to which bubbles are carried by a vertical current, w , before their size makes them smaller than the radius of a 'resonant' bubble. ■, Clean bubbles; +, dirty bubbles. $g = 981 \text{ cm s}^{-2}$; $D = 2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$; $\nu = 1 \times 10^{-2} \text{ cm}^2 \text{ s}^{-1}$, $RT/M = 7.31 \times 10^8 \text{ erg}$, $K = 4.9 \times 10^{-6} \text{ g cm}^{-3} \text{ atm}^{-1}$ appropriate to O_2 , and $\Gamma = 72 \text{ dyn cm}^{-1}$ for clean bubbles, $\Gamma = 36 \text{ dyn cm}^{-1}$ for dirty bubbles.

The presence of Langmuir circulation is marked by the occurrence of wind rows, streaks of foam or debris floating on the surface of the loch and aligned with the wind. To record them, time-lapse ciné films were made of the loch surface above the sounder. The wind rows were unsteady and usually migrated slowly, at 1 or 2 cm s^{-1} across the loch. Care was taken to restrict analysis to periods in which wind rows were clearly visible, depending on suitable lighting conditions. No evidence was found of a relationship between the wind rows and the bubble clouds. A row passing over the sounder did not result in bubble clouds penetrating deeper, and clouds were observed to descend even when no row was present above the sounder. It seems that the large vertical velocities often associated with Langmuir vortices are not dominant or do not penetrate in any coherent way beyond the average depth to which bubbles are seen.

The changes in the cloud structure, deriving partly from advection in the flow through the sonar beam, do, however, apparently reflect the presence or absence of shear. The billow-like clouds are prevalent at the onset of wind or when there is a heat flux into the water, whereas fingers seem to be related to convective conditions when the water in the upper layers moves in a more slab-like fashion⁷. The bubbles do not penetrate to the top of the stable thermocline and fine-scale thermal structures^{6,7} are found in the mixing layer below the level to which bubbles penetrate. This, and the bubble cloud shapes themselves, indicate that the motions carrying bubbles down extend beyond the depth to which bubbles are observed.

The 5–10 min period cloud structures are probably related to the billows which have been detected in the mixing layer, whilst the shorter periods are associated with the frequency of wave breaking above the sonar. Research on these important aspects of air–water interaction continues.

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Occurrence and significance of prist-1-ene in kerogen pyrolysates

A SERIES of acyclic isoprenoid alkanes, of which pristane and phytane are typical, frequently occurs in crude oils, shales, coals, bitumens and so on. It is generally agreed that the primary source of these compounds is the phytyl side chain of chlorophyll^{1,2} although the mechanisms of its incorporation into sediments and its subsequent diagenesis, are not completely understood. At present, little is known about the role of kerogen as a source, or sink, of isoprenoid moieties and we report here that the principal isoprenoid, obtained by the high temperature (600 °C) pyrolysis of kerogens has been identified as prist-1-ene.

Kerogen, which has been defined³ as the fraction of sedimentary organic matter that is insoluble in common organic solvents, may be considered as a chemically-complex polymer believed to arise from the random polymerisation and condensation of biopolymers (such as polysaccharides, peptides and lignins), their degradation products, and other simpler molecules⁴. Kerogen-bound isoprenoid fragments have been reported by several authors; thus degradation by saponification⁵, oxidation^{6–10}, ozonolysis¹¹ and low and high temperature pyrolysis^{12–18} has revealed isoprenoid structures in kerogens.

The use of pyrolysis-gas chromatography (GC) for the elucidation of the structure of kerogens, and coal constituents, is a rapidly developing field¹⁷⁻²², as is its use in their 'typing', that is placing them in groups having similar properties and characteristics²³. Several authors have presented chromatograms of kerogen pyrolysates in which a major non-normal hydrocarbon occurs in the C₁₇-C₁₈ region which does not coelute with pristane^{18,22,24-27}. This component, recently identified as a pristene²⁰ has now been identified as prist-1-ene.

Figure 1 shows three typical pyrograms of solvent-extracted kerogens in which the peak of interest elutes with a retention index of 1,730 (OV-101). This component has been identified as 2,6,10,14-tetramethylpentadec-1-ene (prist-1-ene) on the basis of the following results.

(1) TLC analysis, using silver ion impregnated plates, of a kerogen pyrolysate indicated that the component was olefinic. Hydrogenation of the olefinic mixture resulted in a compound which coeluted with pristane on a high resolution capillary column and whose mass spectrum was the same as that of pristane.

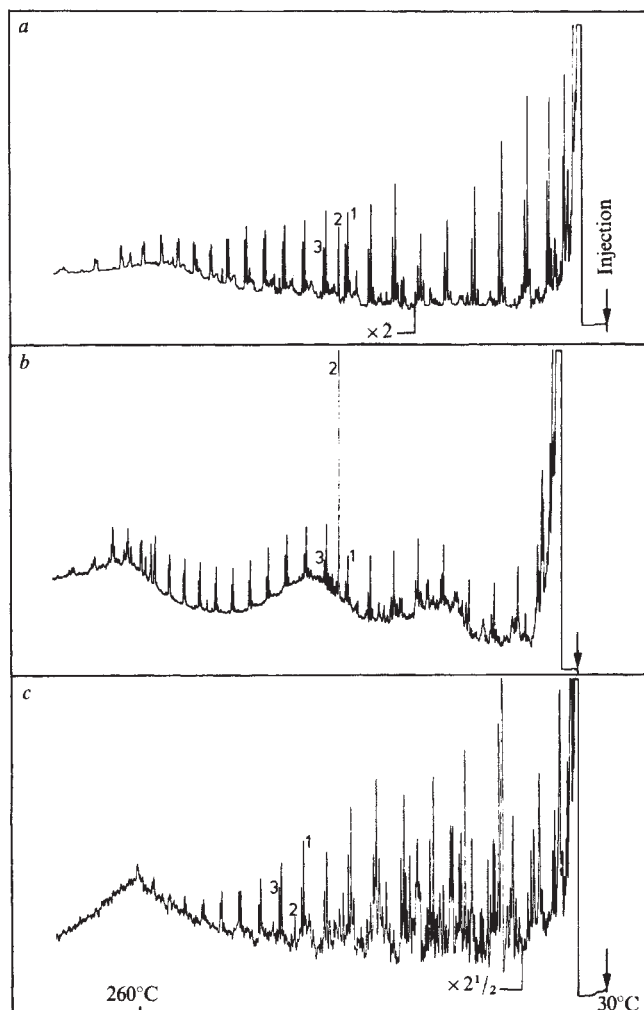


Fig. 1 Pyrograms (600 °C) of kerogens previously extracted thoroughly with dichloromethane. *a*, Algal kerogen from the lacustrine (Eocene) Green River Shale, Colorado, US. *b*, Kerogen from a continental lignitic shale (Miocene), Indonesia. *c*, Kerogen from a lacustrine, poor oil-shale, (Middle Devonian) Caithness, Scotland. The labelled peaks are: 1, *n*-heptadecene; 2, prist-1-ene; 3, *n*-octadecane. (Conditions: pyrolysis at 600 °C in nitrogen, followed by gas chromatography on 20m (*a* and *b*) and 25m (*c*) capillary columns coated with OV-101. Oven temperature programmed from 30 to 260 °C at 4 °C min⁻¹).

(2) Coinjection of the kerogen pyrolysate with a synthetic mixture contain prist-2-ene and prist-1-ene, on a high-resolution capillary column indicated that the isoprenoid component of the kerogen pyrolysate was prist-1-ene. Although coinjection was performed on one liquid phase only, the very high efficiency of the OV-101 capillary column (~80,000 effective theoretical plates) easily separated the two pristene isomers. This was confirmed by the GC analysis, under similar conditions, of prist-1-ene and prist-2-ene synthesised, as a mixture, from 2,6,10,14-tetramethylpentadecan-2-ol, through the *p*-tolylsulphonyl derivative²⁸. The proportion of the two isomers in the synthetic mixture was confirmed by NMR analysis.

(3) Pyrolysis-GC-MS of the kerogens, and GC-MS analysis of a trapped kerogen pyrolysate, and authentic prist-1-ene (in (2)) all gave similar mass spectra for the compound in question (Fig. 2).

Note that on the 'boiling point' liquid phase (OV-101) used for most of this GC work the pristenes elute later than pristane. This differs from the behaviour of unbranched hydrocarbon chains where *n*-alkenes elute before *n*-alkanes of equivalent carbon number²⁹. The Kovats retention indices³⁰ of the respective C₁₉ hydrocarbons, determined by temperature-programming an OV-101 capillary column, were: pristane: 1,709; prist-1-ene: 1,730; prist-2-ene: 1,742.

Pyrolysis (600 °C) of more than 150 kerogens²⁷ indicated that prist-1-ene occurred in about 50 of the pyrolysates and was, surprisingly, the only acyclic isoprenoid hydrocarbon to appear (except for a few which contained traces of prist-2-ene). There is, however, no readily discernible relationship between the occurrence of prist-1-ene in a kerogen pyrolysate and the kerogen type as defined by Tissot and Welte²³. Analysis of separated coal maceral kerogens has indicated the apparent absence of isoprenoid moieties in alginite kerogen pyrolysates²⁷ although other hydrogen-rich (type 1) kerogens, such as Green River and Messel shale kerogen do contain this isoprenoid alkene.²⁰ The abundance of prist-1-ene in the pyrograms of the kerogens that we have analysed so far, varies from its apparent absence to a relative abundance of ~10 times that of the C₁₇ *n*-alkane. It has been observed also that the relative abundance of pristene, with respect to, for example, the *n*-C₁₇ alkane, in any kerogen pyrolysate decreases with increasing pyrolysis temperature.

In contrast to the simple distribution of isoprenoid hydrocarbons in the pyrograms of these ancient kerogens, pyrolysis of synthetic melanoidins, (dark-coloured, acidic, polymers produced by the condensation of amino acids and carbohydrates) containing chemically-bound phytol produces, in addition to prist-1-ene, a wide variety of isoprenoid alkenes and alkanes with carbon numbers ranging from 14 to 20 (unpublished data). Chlorophyll and pheophytin also produce prist-1-ene on pyrolysis, but the pyrograms are dominated by a suite of other isoprenoid components. Mixtures of isoprenoid alkenes (pristenes and phytene) have also been observed in the pyrolysates of recent algal mat kerogens¹⁶. These results suggest that during early diagenesis isoprenoid-kerogen associations differ from those in ancient kerogens.

Although prist-1-ene occurs naturally in marine zooplankton³¹ and although several schemes have been proposed for the diagenetic conversion and assimilation of phytol and its derivatives in sediments³²⁻³⁵ virtually nothing is known of the mechanisms by which isoprenoid moieties are incorporated into accreting heteropolycondensate (kerogen) molecules. The importance of assessing the amount, and type of bonding of isoprenoid structures in kerogens is clear when one considers that kerogen may be the source of much of the isoprenoid alkane fraction obtained during the artificial maturation^{14,15} of sedimentary organic matter and also by catagenesis when it is deeply buried.

As the products of flash pyrolysis (600 °C) are not in thermodynamic equilibrium, the appearance of only one isoprenoid component in kerogen pyrolysates indicates that kerogen-isoprenoid bond types are few. Van der Berg *et al.*¹¹ using low

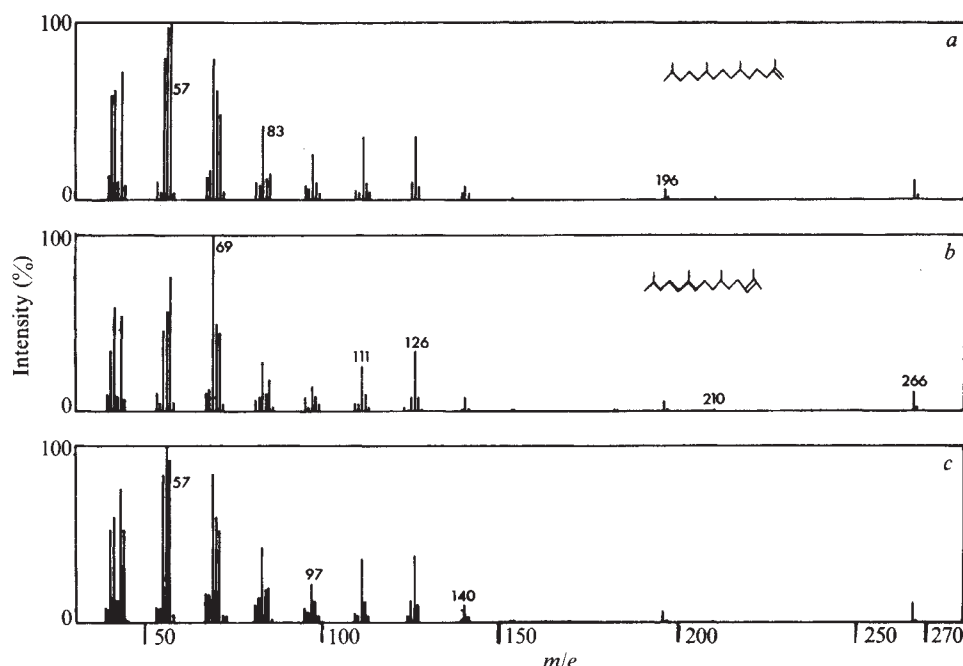


Fig. 2 Mass spectra of *a*, authentic 2,6,10,14-tetramethylpentadec-1-ene (prist-1-ene); *b*, 2,6,10,14-tetramethylpentadec-2-ene (prist-2-ene); and *c*, prist-1-ene in pyrolysed Green River shale kerogen. (Spectra, taken at 70 eV, show ions of >2% intensity related to the base peak.)

temperature ozonolysis of Messel shale kerogen, considered that isoprenoid structures were attached to the kerogen with olefinic linkages. It is possible, however, that the reduction of double bonds during diagenesis results in saturated groups being attached to the kerogen nucleus. If this is so then saturated isoprenoid chains in a kerogen may be derived either by saturation of an unsaturated isoprenoid fragment bound to the kerogen or by direct incorporation of a saturated isoprenoid fragment. It is well known, for example, that the short term diagenesis of chlorophyll, and phytol, produces several saturated isoprenoids including dihydrophytol and phytanic acid^{32,33}; it seems reasonable to assume that these species could be bound into an accreting kerogen, particularly in the light of the rapid incorporation of fatty alcohols and acids into synthetic melanoidins with the concomitant expulsion of carbon dioxide²⁷. It is probable that during early diagenesis functionalised isoprenoid species can link to a kerogen matrix by, for example, ester linkages but the stability of ester groups, or others, in kerogens during diagenesis, and subsequent maturation, is not completely understood.

The almost exclusive presence of prist-1-ene in kerogen pyrolysates can be explained in terms of the abstraction of the tertiary hydrogen atom of the isoprenoid chain nearest to the kerogen, followed by rearrangement and release of the isoprenoid moiety. While the possibility of a radical mechanism for pristene formation cannot be excluded, rearrangement reactions in pyrolysis are favoured by the presence of tertiary hydrogen atoms and adjacent functional groups^{36,37}. In a poly-functional molecule such as kerogen many such active sites may be expected.

While little is known of the details of pyrolysis reaction mechanisms, there is little doubt that pyrolysis-GC is a versatile and rapid tool for determining the presence of specific structural elements in kerogens. In particular, the application of this technique to the investigation of the nature and distribution of biomarker fragments (such as acyclic isoprenoids, di- and tri-terpenoids, and steroids) attached to geopolymers such as humic substances and kerogens may be important in assessing the relative contribution of kerogen-derived compared with biolipid-derived soluble organic species in rock extracts. This is likely to be important for the correct interpretation of changes in the amount, type and distribution of hydrocarbons, and so on in rock extracts with parameters such as depth of burial and organic matter type: these are questions of fundamental importance in source rock geochemistry.

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Microwave measurements of the absolute values of absorption by water vapour in the atmosphere

MEASUREMENT of the absolute value of absorption by water vapour at microwave frequencies is difficult because the effect is so small. Far in the wings of the absorption lines, in the so-called 'windows' of the spectrum, it is especially difficult to achieve high accuracy in the free atmosphere. But it is in these windows that the behaviour of the absorption is important from both applied and scientific points of view. Satellite communications, remote sensing of the atmosphere, and radioastronomy, are all influenced by this behaviour. Measurements on an Earth-space path are reported here; the results indicate a nonlinear relationship between absorption and water-vapour content.

Terrestrial transmission measurements at 70 and 80 GHz reported previously¹ showed that the absolute value of the absorption by water vapour, at typical vapour densities, is three or four times larger than predicted² for these frequencies. Some nonlinearity of absorption with vapour density was also observed. The empirical factor, believed to be associated with the water-vapour 'continuum', has been used in computations of absorption^{3,4}. The cause of the continuum absorption is still debatable although it may be due to clustering of the water molecules⁵. Unexplained large absorptions in the windows of the infrared have been measured⁶. Recent laboratory measurements⁷ at 230 GHz showed larger absorption than expected, and a quadratic dependence of absorption on the partial pressure of water vapour was also observed.

In developing instruments for passively remote-sensing the troposphere, we are also investigating the behaviour of absorption by water vapour on Earth-space paths. In this case, the measurements are radiometric, at frequencies of 20.6 and 31.65 GHz; the former is on the side of an absorption line, the latter in a 'window'. The dual-channel radiometer is located at the US National Weather Service Forecast Office, Denver, Colorado, where radiosondes are launched twice daily. In this way, total emission (and hence total absorption) measured by the radiometers, is compared with the integrated (or precipitable) vapour measured by the radiosondes on a systematic basis.

The measurements are made on clear, cloudless days by scanning an antenna, of equal beamwidth at the two frequencies, in elevation angle. The brightness temperatures are converted to absorption which is then plotted against air mass as in the

example shown in Fig. 1. At both frequencies, the absorption is found to behave linearly to beyond seven air masses (elevation angle 9°). In this way, accurate values of total absorption in the zenith are obtained.

Zenith absorptions at both frequencies measured during 1978 are shown in Fig. 2. Although Colorado has a relatively dry climate, precipitable vapour up to about 2.5 cm is encountered. Fits to a straight line, and to curves of higher power, have been made to the data. For the linear fit, the standard error of measurement is 0.031 dB at 20.6 GHz and 0.019 dB at 31.65 GHz. However, fitting with a polynomial results in standard errors of 0.028 and 0.015 at 20.6 and 31.65 GHz respectively. The polynomial fits therefore show an improvement over the linear fits, especially at 31.65 GHz in the transmission 'window' where the continuum is most influential. The scatter in the data is caused in part by lack of horizontal homogeneity in the atmosphere, and in part by non-coincidence

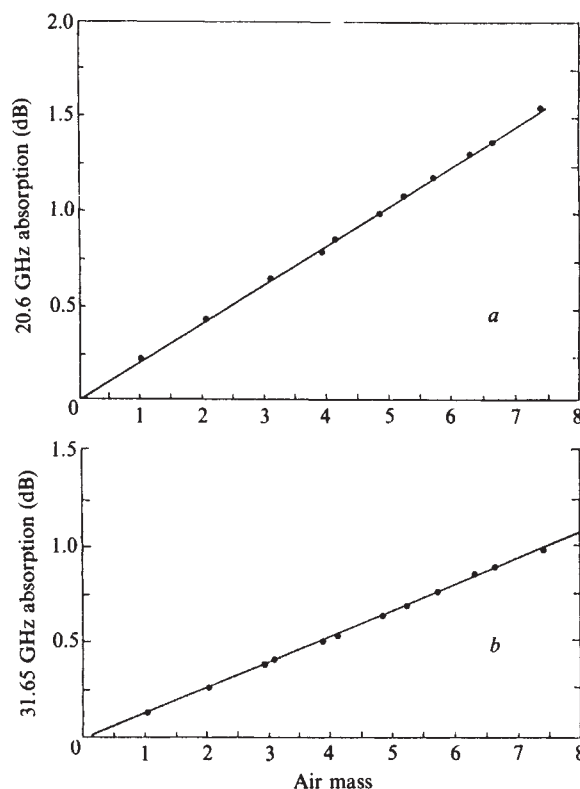


Fig. 1 Example of measurement of total absorption through the atmosphere at 20.6 GHz (a) and 31.65 GHz (b), plotted as a function of air mass.

in radiosonde flight time and absorption measurement; the latter contributes about 1 mm r.m.s. error on the abscissa in Fig. 2. The error introduced by the radiometric instruments is <0.01 dB which is about one-tenth of the maximum peak-to-peak deviations. The accuracy of humidity measurement by radiosonde is known to be questionable; the hygrometers are especially erratic at relative humidities below 20%. Estimates place the accuracy of measurement of precipitable vapour at about 1.5 mm which does not materially alter the conclusion regarding the nonlinear fit in Fig. 2.

The behaviour of the 31.65 GHz data of Fig. 2b at low values of vapour is curious; the total absorption decreases slightly with increasing vapour. This effect is caused by the inverse dependence of oxygen absorption on temperature, that is, as low precipitable vapour occurs during cool temperatures, the

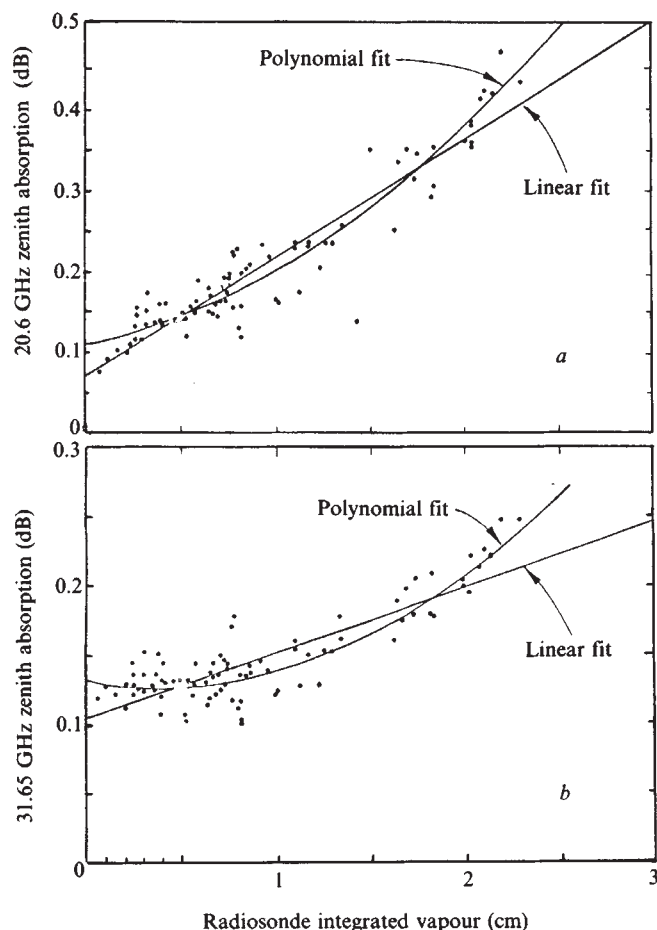


Fig. 2 Absorption in the zenith at 20.6 GHz (a) and 31.65 GHz (b) plotted against precipitable water vapour measured by radiosondes; standard errors at 20.6 GHz and 31.65 GHz are 0.031 and 0.019, 0.028 and 0.015 (dB) for the linear and polynomial fits respectively. The slight downward trend in (b), from 0 to 0.5 cm, is caused by an inverse temperature effect in absorption by oxygen.

absorption by oxygen is higher than during high-humidity conditions. The equation describing the nonlinear fit in Fig. 2b is:

$$A = 0.13 - 0.026V + 0.032V^2$$

We conclude that microwave absorption in the transmission windows of the atmosphere has a significant quadratic dependence on the water-vapour content, as opposed to the linear relationship assumed in the past. This matter is of importance in remotely sensing water vapour in the atmosphere, because if the absorption process is not fully understood, accurate values of vapour are not retrieved from radiometric observations; it is also of interest in studying phase changes involved in formation of clouds.

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Global inventory of natural and anthropogenic emissions of trace metals to the atmosphere

FEW (if any) recent studies on the atmospheric cycle of trace metals have considered the flux of the metals into the atmosphere on a global scale. Information on worldwide emissions is needed to assess the transboundary movement of pollutant metals and to validate models of the global atmospheric circulation patterns. The worldwide inventories of the natural and anthropogenic sources and emissions of airborne cadmium, copper, lead, nickel and zinc are presented here. The data summarised here are taken from very detailed studies published elsewhere^{1–5}.

Worldwide emissions of the five metals from natural sources are shown in Table 1. The data presented were derived indirectly by considering only the quantity of aerosols released from each source and the emission factor applicable to each particular process. An obvious weakness in estimating the relative contribution from each source derives from discrepancies in the reported particulate emission rates (as indicated by the ranges in Table 1). These discrepancies reflect the uncertainty in estimates of global trace metal emissions; variability in the emission factors used in the calculations are unlikely to change the emission rate from any of the sources by an order of magnitude. The data in Table 1, thus, are order of magnitude estimates to be refined as more definitive data become available on the particulate emission rates. It should be emphasised that Table 1 shows the present-day emission rates which could conceivably include retransmission of metals of anthropogenic origin.

Eroded soil particles generally account for ~60–80% of the Cu, Pb, Ni and Zn (but only for ~6% of the Cd) emitted from natural sources (Table 1). Volcanic emissions account for ~60% of the Cd from natural sources, reflecting the abnormally high enrichment of the metal in volcanogenic aerosols. Volcanoes, on the other hand, account for 15–25% of the other four metals released from natural sources (Table 1). Vegetative exudates clearly represent an important natural source of metals in the pre-Mesolithic terrestrial atmosphere. The estimate that plant exudates account for nearly 20% of zinc released from natural sources is in accord with the recent observation that plants release significant amounts of zinc into the atmosphere^{6,7}. Forest fires and seasalt sprays represent only minor sources of trace metals in the global atmosphere, generally accounting for <10% of the natural trace metal emissions. Natural rock degassing has been suggested as an important source of airborne trace metals^{8,9} but the contribution of such a source to the atmospheric trace metal burden remains to be quantified.

A comparison of Tables 1 and 2 shows that the annual anthropogenic emissions of Cd and Pb exceed the natural rates by well over an order of magnitude. The current anthropogenic emissions exceed the natural rates for Cu, Ni and Zn by ~300%, 200% and 700% respectively. Non-ferrous metal production and use account for 43% (Zn) to 67% (Cd) of the pollutant Cd, Cu and Zn released annually to the atmosphere. On the other hand, the automotive combustion of leaded gasoline and nickeliferous diesel oil account for about 60% of the pollutant Pb and Ni emissions (Table 2). The extraction and industrial applications account for a little more than 20% of the latter two metals emitted from pollutant sources. Table 2 also clearly

Table 1 Worldwide emissions of trace metals from natural sources

Source	Global production ($\times 10^9$ kg yr $^{-1}$)*	Worldwide annual emissions ($\times 10^6$ kg)				
		Cd	Cu	Ni	Pb	Zn
Windblow dusts†	500(6–1100)	0.1(0.001–0.22)	12(0.14–26)	20(0.24–44)	16(0.19–35)	25(0.30–55)
Forest fires‡	36(2–200)	0.012(0.001–0.07)	0.3(0.025–1.7)	0.6(0.05–3.3)	0.5(0.04–2.8)	2.1(0.18–12)
Volcanogenic particles§	10(6.5–150)	0.52(0.3–7.8)	3.6(2.3–54)	3.8(2.4–56)	6.4(4.2–96)	7.0(4.6–105)
Vegetation	75(75–1,000)	0.2(0.2–2.7)	2.5(2.5–33)	1.6(1.6–21)	1.6(1.6–21)	9.4(9.4–125)
Seasalt sprays¶	1,000(300–2,000)	~0.001	0.08(0.02–0.2)	0.04(0.01–0.08)	0.02(0.01–0.05)	0.01(0.004–0.02)
Total**		0.83	18.5	26.0	24.5	43.5

* Most acceptable values are given, with the range in the literature values shown in brackets. See ref. 21 for review of airborne particulate emissions.

† The emission factors used in these calculations correspond to the average concentrations of the trace metals in soils^{22–24}.

‡ The emission factors assume average ash content of forest trees and foliage to be 4% and the average Cd, Cu, Pb, Ni and Zn contents of the ash to be 8, 200, 450, 200 and 1,450 $\mu\text{g g}^{-1}$, respectively^{25,26}.

§ The emission factors entail the average crustal abundances ($\mu\text{g g}^{-1}$): Cd, 0.2; Cu, 24; Pb, 16; Ni, 75; and Zn, 70 (refs 24 and 27), and 260-, 15-, 40-, 5- and 10-fold enrichments of Cd, Cu, Pb, Ni and Zn in the volcanogenic particles^{28,29}.

|| The emission factors assume average ash content of vegetative exudates to be 11% and the metal concentrations in the ash residue to be 25, 300, 200, 25 and 1,140 $\mu\text{g g}^{-1}$ for Cd, Cu, Pb, Ni and Zn, respectively.

¶ Recent data on Cd, Cu, Ni, Pb and Zn concentrations in surface ocean waters of 5.8, 150, 210, 12 and 8.5 ng l^{-1} (refs 30–33 and Schaule and Patterson, personal communication, were combined with 200-, 550-, 200-, 2,000- and 1,400-fold enrichments of Cd, Cu, Ni, Pb and Zn, respectively, in the atmospheric seasalt particles^{34–36} to derive the pertinent emission factors.

** The total values given are for the most acceptable production figures.

Table 2 Worldwide anthropogenic emissions of trace metals during 1975

	Global production or consumption (10^9 kg yr $^{-1}$)	Trace metal emissions ($\times 10^6$ kg yr $^{-1}$)†				
		Cd‡	Cu‡	Pb§	Ni	Zn‡
Mining, non-ferrous metals	16	0.002	0.8	8.2		1.6
Primary non-ferrous metal production						
Cd	0.0017	0.11				
Cu	7.9	1.6	19.7	27	1.5	6.6
Pb	4.0	0.20	0.29	31	0.34	0.44
Ni	0.8			2.5	7.2¶	0.68
Zn	5.6	2.8	0.78	16	0.36	99
Secondary non-ferrous metal production	4.0	0.60	0.33	0.77	0.2	9.5
Iron and steel production	1,300	0.07	5.9	50	1.2	35
Industrial applications	–	0.05	4.9	7.4	1.9	26
Coal combustion	3,100	0.06	4.7	14	0.66	15
Oil (including gasoline) combustion	2,800	0.003	0.74	273	27	0.07
Wood combustion	640	0.2	12	4.5	3.0	75
Waste incineration	1,500	1.4	5.3	8.9	3.4	37
Manufacture, phosphate fertilisers	118	0.21	0.6	0.05	0.6	1.8
Miscellaneous	–	–	–	5.9	–	6.7
Total		7.3	56	449	47	314

* The sources of the consumption and production figures are given in refs 1–5.

† The emission factors used to derive the data shown come from a wide variety of sources. The core references (in terms of the emission factors) include: Cd^{37–39}; Cu^{39,40}; Pb^{12,14,41}; Ni^{38,42} and Zn^{39,42,43}.

‡ The emission data listed for Cd, Cu and Zn are taken from refs 5, 4 and 3, respectively.

§ Modified from the inventory given in ref. 1.

|| Adapted from the inventory presented in ref. 2.

¶ Includes emissions from mining operations.

Table 3 All-time worldwide consumption and anthropogenic emissions of trace metals

	Global metal consumption ($\times 10^9$ kg)*					Global metal emissions ($\times 10^6$ kg)				
	Cd	Cu	Pb	Ni	Zn	Cd†	Cu	Pb	Ni	Zn
Pre-1850	–	45	55	–	50	63	319	2,420‡	–	2,804
1850–1900	–	13	25	0.2	15	19	92	1,100‡	12	841
1901–10	–	7.5	10.7	0.14	7.0	8.9	53	471‡	8.2	392
1911–20	0.001	11.3	11.2	0.35	8.8	11	80	493‡	21	493
1921–30	0.007	13.5	14.2	0.36	11.1	14	96	1,120‡	21	622
1931–40	0.026	16.3	14.6	0.83	13.3	17	116	1,639	49	746
1941–50	0.048	23.8	14.9	1.37	17.1	22	169	1,672	80	959
1951–60	0.084	32.4	24	2.38	27	34	230	2,694	140	1,514
1961–70	0.14	61.4	33	4.37	42.3	54	435	3,704	257	2,372
1971–80	0.15	82.5	38	7.07	58	74	585	4,265	415	3,252
Total (all time)	0.50	307	241	17	250	316	2,175	19,578	1,003	13,995

* The pre-1850 production figures for Pb and Zn are derived from refs 1 and 44. The historical production data for the decades between 1900 and 1980 were either taken directly from ref. 16 or compiled from the data listed in ref. 45. The 1850–1900 data are from ref. 46 or ref. 47 (Ni).

† These data are based on the ratio of Cd emission to Zn production during the 1975 base year (Table 2).

‡ The fact that plumbiferous gasoline was introduced only in 1923 was taken into account in deriving these values. Thus, the effect of gasoline contribution was subtracted from the global Pb emission before normalising to the 1975 base year.

§ The 1979–80 production figures were extrapolated from the 1970–77 historical data.

implicates waste incineration as an important source of metalliferous aerosols, and may well be the principal source of metal aerosols in some urban areas as suggested by Greenberg *et al.*¹⁰. Significant contributions to the atmospheric trace metal burden also come from the iron and steel industry and coal combustion.

The present estimate of pollutant lead emission is higher than the $260 \times 10^6 \text{ kg yr}^{-1}$ reported by Guicherit¹¹. His estimate seems low considering that the most comprehensive study of Pb emissions in the US (ref. 12) puts the anthropogenic figures at $162 \times 10^6 \text{ kg yr}^{-1}$. In relation to the fraction of lead consumed and produced, the global emission rate should at least be twice that of the US, that is $>324 \times 10^6 \text{ kg yr}^{-1}$. Heindryckx *et al.*¹³ estimated the global Cd emissions to be $7.3 \times 10^6 \text{ kg yr}^{-1}$, in excellent agreement with what is reported in Table 2. Worldwide emission rates for Cu, Ni and Zn have apparently not been reported previously. In the National Academy of Sciences Report¹⁴, the worldwide emission of Cu from coal combustion and the copper industry was put at $16.3 \times 10^6 \text{ kg yr}^{-1}$, a figure in reasonable agreement with the data listed in Table 2.

There has been no previous attempt to estimate the all-time amounts of the trace metals released into the atmosphere from anthropogenic sources. To obtain the historical rates of man-induced emissions, we assume that the annual emission of a given metal is proportional to the amount of that metal produced/consumed per annum. This is not unreasonable. First, the principal contributors to the atmospheric trace metal burden (notably the extraction and use of non-ferrous metals, iron and steel production and fossil fuel combustion) tend to mirror the state of the global economy with the result that the historical growth rates for these commodities tend to be quite similar (see Robinson¹⁵ and Dowding¹⁶). Furthermore, it has already been noted that a large fraction of the trace metal emission is associated with non-ferrous metal production and usage (Table 2). By combining the emission-to-production ratios (derived from data in Table 2) with the historical production figures for the metals shown in Table 3, the historical rates of the trace metal emissions from anthropogenic sources have been calculated (Table 3). The all-time amounts of the metals released from pollutant sources are also given in Table 3.

As expected, sharp increases in the man-induced release of trace metals have occurred since the beginning of this century. It is encouraging that the ratios of the 1901–10 to the 1971–80 emissions of metals calculated from the data in Table 3 are consistent with the historical changes in rates of trace metal deposition observed in polar ice cores in the Northern Hemisphere^{17–19}. The recent report²⁰ that there have been no significant elevations of trace metals in polar snow from the Southern Hemisphere underlines the important fact that the bulk of man-made emissions occur in the Northern Hemisphere.

It is estimated that the total (all-time) amounts of pollutant Cd, Cu, Pb, Ni and Zn that have been dispersed to the world ecosystems through the atmosphere are $0.32 \times 10^9 \text{ kg}$, $2.2 \times 10^9 \text{ kg}$, $20 \times 10^9 \text{ kg}$, $1.0 \times 10^9 \text{ kg}$ and $14 \times 10^9 \text{ kg}$, respectively. Also, most of the all-time stock of each metal produced may be expected to be wasted in the terrestrial biosphere. The redistribution of such massive amounts of trace metals at the Earth's surface merely emphasises the current impacts of human activities on the global cycles of many elements.

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Nickel and copper accumulation as essential elements in 10-Å manganite of deep-sea manganese nodules

MANY ferromanganese minerals have been found in marine manganese deposits. There have been terminological and intrinsic problems in accurately defining the constituents of manganese nodules¹, but 10-Å manganite and $\delta\text{-MnO}_2$ which are often compared with todorokite and birnessite seem to be the only major constituents of marine manganese nodules and crusts. [Note that birnessite is a natural species of $\delta\text{-MnO}_2$ of four-line form, whereas marine $\delta\text{-MnO}_2$ is two-line form, so marine and terrestrial $\delta\text{-MnO}_2$ should not be compared directly with each other.] However, there have been few experimental studies of the mechanism of accumulation and the crystallochemistry of these elements in 10-Å manganite of manganese nodules, although many experimental studies of adsorption of metals to natural and synthetic manganese and iron oxides have been made^{2,3}. I have now investigated the mechanism of accumulation of nickel and copper in manganese nodules by synthesising 10-Å manganite and comparing the composition with that shown by microscopic and electron microprobe analyses of marine 10-Å manganite.

Previously reported geochemical features of marine manganese nodules, such as inter-element relationships,

Table 1 Characteristics of nodule constituents

Characteristics	10-Å manganite phase	δ -MnO ₂ phase
Optical properties (reflecting microscope)		
colour	light grey	dark grey
reflectivity	high	low
anisotropism	strong	none
hardness (VHN)*	high (52–112, mean 82)	low (10–24, mean 17)
internal reflection	none	none
Occurrence	deep-sea manganese nodules (chiefly bottom surface)	deep-sea manganese nodules (chiefly top surface)
	shallow-water manganese nodules	crust or coating of rocks from topographic highs
Texture (reflecting microscope)	conformable thin layer	stratification (sometimes columnar)
	dendritic (cauliflower-like cusps)	
	network crack filling cementing of clastics	
Chemistry (microprobe analysis)	rich in Mn Mn: 30–50 wt % Fe: 0–2 Ni: 1–3 Cu: 1–2 Co: 0–0.4 Si: 0–1	rich in Mn, Fe and Si Mn: 10–30 wt % Fe: 11–18 Ni: 0–0.8 Cu: 0–0.8 Co: 0.3–0.6 Si: 1–8
Mineralogy	monomineralic (10-Å manganite)	mixture of some minerals—two-line form δ -MnO ₂ , amorphous iron hydroxide, and minute detrital minerals, such as quartz, plagioclase, zeolite and clay minerals

* VHN, Vicker's hardness number.

heterogeneous distribution of elements in individual nodules, compositional variation with the occurrence, and morphological and mineralogical controls on metal contents, are systematically explained by the chemical and mineralogical characteristics of the two phases of two-line form δ -MnO₂ and 10-Å manganite in manganese nodules² as shown (Table 1). The δ -MnO₂ phase consists of submicroscopic mixture of colloidal particles of two-line form δ -MnO₂, amorphous iron hydroxide, and detrital minerals, whereas the 10-Å manganite phase consists of a monomineralic manganese oxide with minor amounts of nickel, copper, magnesium and other metal elements.

Nickel and copper are at least six times more concentrated in the 10-Å manganite phase than in the δ -MnO₂ phase^{3,4}. Therefore, most of the copper and nickel in manganese nodules is attributable to the 10-Å manganite phase. Glasby⁵ and Burns and Burns⁶ suggested that there may be a maximum limit in the concentration of copper and nickel or in their ratio to manganese. This fact is probably related to the mechanism of accumulation and minor metal elements in manganese nodules.

The first synthesis of 10-Å manganite was carried out by Wadsley⁹, and Buser and Grütter¹⁰ compared the synthesised compound with the nodule constituents. McKenzie¹¹ summarised the experimental procedure for synthesis of 10-Å manganite, but he did not refer to the marine manganese minerals although he cited todorokite as equivalent terrestrial mineral species.

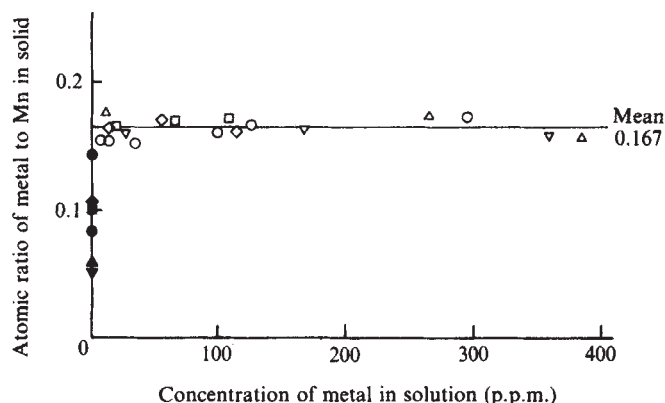


Fig. 1 The ratios of amounts of incorporated metal-to-manganese in reaction products plotted against the metal concentration in solution, showing no dependency of incorporation on the kind or concentration of these metal ions. Solid symbols indicate that the 7-Å reflection remains in the X-ray pattern of the product, and open symbols indicate a complete change into 10-Å manganite. ○, NiCl₂; △, CuCl₂; ▽, CoCl₂; □, CaCl₂; ◇, MgCl₂.

In the present study, 10-Å manganite was synthesised from manganese nitrate with a high reproducibility by a modified method from McKenzie's. The primary compound, identified as δ -MnO₂ (four-line form) by the X-ray pattern, was synthesised by mixing manganese nitrate and sodium hydroxide solutions in oxygenated conditions. This primary compound is not stable in the presence of certain ions such as Ca²⁺, Mg²⁺, Ni²⁺, Cu²⁺, Co²⁺, and Zn²⁺, and it transforms rapidly into 10-Å manganite-equivalent through the irreversible interactions with the ions, resulting in the incorporation of these ions and the release of equivalent amount of Na⁺ into solutions. The amount of metals incorporated into the 10-Å manganite is markedly constant irrespective of the concentration and kind of the ions (Fig. 1). The atomic ratio of the incorporated metals to manganese in 10-Å manganite is ~0.167 (~1/6), and the ratio of released sodium to manganese in the solution is ~0.318 (~1/3) (Table 2).

Thus the ions Ca²⁺, Mg²⁺, Ni²⁺, Cu²⁺, Co²⁺, and Zn²⁺ are apparently located in the definite cation sites of the crystal structure of 10-Å manganite. Cations larger than Ca²⁺ ($r = 0.99$ Å) in effective ionic radius, such as Ba²⁺ and Pb²⁺, are not incorporated in these sites and cannot be essential elements of 10-Å manganite.

Further experiments were carried out to establish the order of preference of incorporation of various elements into the cation site of 10-Å manganite. Ni²⁺ is incorporated into 10-Å manganite with two or three orders of magnitude preference over Mg²⁺ and Ca²⁺. Furthermore, gradual enrichment of nickel was found in 10-Å manganite during the reaction (at constant stoichiometric composition) in which 10-Å manganite was formed in the presence of mixed solutions of Ni²⁺ and Mg²⁺ (or Ca²⁺). This order of preference for incorporation of metal ions into the cation sites of 10 Å manganite probably results from the difference of ionic radii of metal ions. Large metal ions such as Ca²⁺ ($r = 0.99$ Å) or smaller ions such as Mg²⁺ ($r = 0.60$ Å) may be less stable than Ni²⁺ ($r = 0.69$ Å) and probably Cu²⁺ ($r = 0.66$ Å), in the cation sites of 10-Å manganite.

These experimental results also explain the maximum limit to the concentration of nickel and copper⁶ and the maximum ratio of these elements to manganese⁵ found in manganese nodules. Figure 2 shows the maximum ratio of Cu+Ni+Co to Mn (maximum atomic ratio ~0.15) in the 10-Å manganite phase. Different values of the ratio (Cu+Ni+Co) to Mn can be explained with the compensation by calcium and magnesium in the 10-Å manganite phase of manganese nodules, because the calculated atomic ratio (Ni+Cu+Mg+Ca)/Mn in this phase of deep-sea manganese nodules from the Central Pacific Basin on the basis of the electron microprobe analyses, is ~0.17 ±

0.012 s.d. The value of 0.17 ($\sim 1/6$) is close to that of synthetic 10-Å manganite (Fig. 2 and Table 2).

The inter-element relationship between nickel/copper and manganese, which has been invoked to explain the mechanism of accumulation of these minor metal elements by surface adsorption on manganese hydroxides^{12,13}, is, rather, evidence of isomorphic substitution which controls the stoichiometric composition of marine 10-Å manganite which sustain nickel and copper as essential elements in the crystal structure. Assumed post-depositional enrichment of nickel and copper may take place in the 10-Å manganite phase through the isomorphic substitution of Ca or Mg with Ni and Cu between 10-Å manganite and sediment interstitial water. However, the concept of post-depositional enrichment of nickel and copper in 10-Å manganite should be investigated in more detail in terms of synthesis experiments of related manganese compound and comparative mineralogical and chemical analyses of associated submarine sediment and interstitial water with manganese nodules.

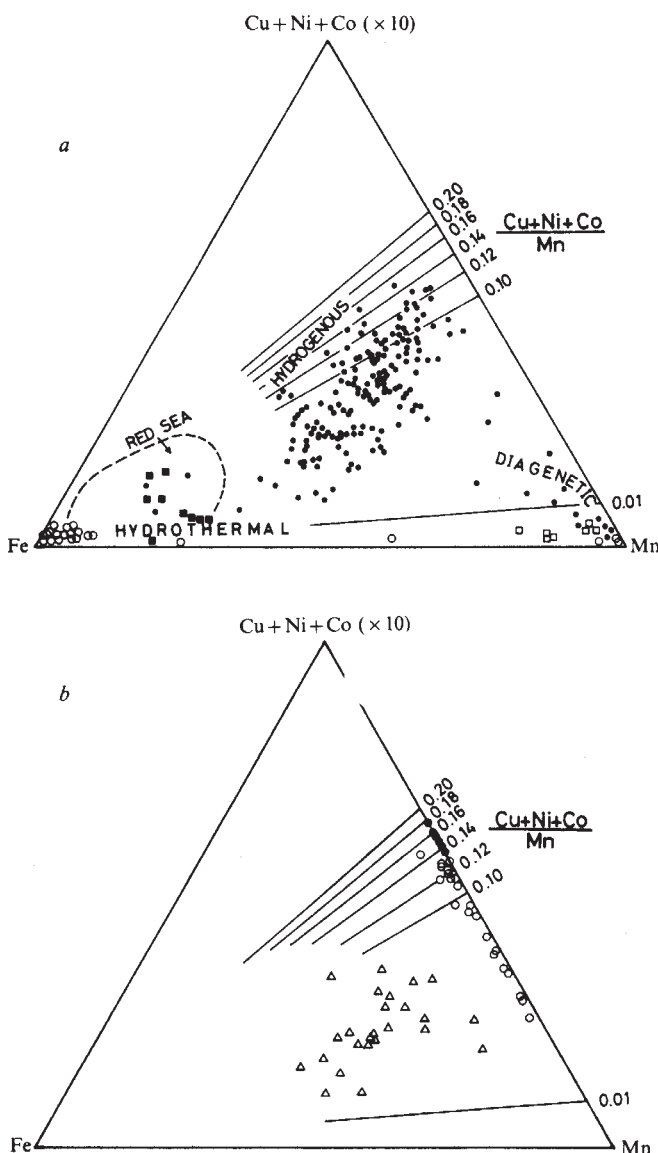


Fig. 2 *a*, Ratio Fe/Mn/(Cu+Ni+Co) in ferromanganese deposits. (From Bonnatti *et al.*¹⁴). Pacific nodules, ●; deposit from Loch Fyne, □; Thera, Stronboli, Afar and AMPH D2, ○; East Pacific Rise, ■. *b*, Ratio Fe/Mn/(Cu+Ni+Co) in the two constituents of nodules. Most of all plots for the 'hydrogenous' deposits in (*a*) fall on the region between the 10-Å manganite and δ -MnO₂ phases. δ -MnO₂ phase (deep-sea nodules), △; 10-Å manganite phase (deep-sea nodules), ○; 10-Å manganite, syn., ● (From Usui *et al.*⁴).

Table 2 Ratio of incorporated metals to manganese in the synthetic 10-Å manganite derived from various metal chloride solutions

	R/Mn ratio wt	R/Mn ratio mol	Mn release mol	Na release mol	R sorb. (Mn rel. + 2Na rel.)
Ni (13)	0.168	0.157	0.004	0.312	91%
Co (6)	0.189	0.176	0.048	0.300	89
Cu (6)	0.206	0.178	0.069	0.303	81
Ca (4)	0.119	0.175	0.000	0.330	102
Mg (3)	0.072	0.162	0.000	0.330	99
Mean:		0.167	0.023	0.318	91

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Santa Catharina and the origin of cloudy taenite in meteorites

CLOUDY taenite appears as a dark-etching rim that occurs at the high nickel border of taenite fields in iron meteorites and mesosiderites. Transmission electron microscopy (TEM) shows it to consist of a foam-like association of interpenetrating single crystals of taenite and kamacite when well developed¹ with an orientation relationship between those of the Kurdjumow-Sachs and the Nishiyama². It occurs between compositional limits that have been reported as ~ 25 –40% Ni¹ and 30–40% Ni³. Observed taenite particle sizes range from 0.4 μ m in the Estherville mesosiderite¹ to considerably less than 0.1 μ m for many iron meteorites (unpublished data). The mechanism of cloudy taenite formation has not been previously explained, although it is believed² to be a phenomenon related to the Invar effect, which occurs in Fe–35% Ni (Invar) and several other alloy systems. Santa Catharina is a wholly taenitic anomalous iron meteorite that contains 35% Ni and 0.6% Co (ref. 4). This composition, which is very similar to that of Invar, places it well inside the range where cloudy taenite is known to form. The optical microstructure of Santa Catharina suggested that the matrix phase of the entire meteorite may consist of cloudy taenite, so I have now examined it by TEM and the results are reported here.

The electron micrographs (Fig. 1*a*) show a fine duplex structure with a particle size of ~ 10 nm present both in the matrix

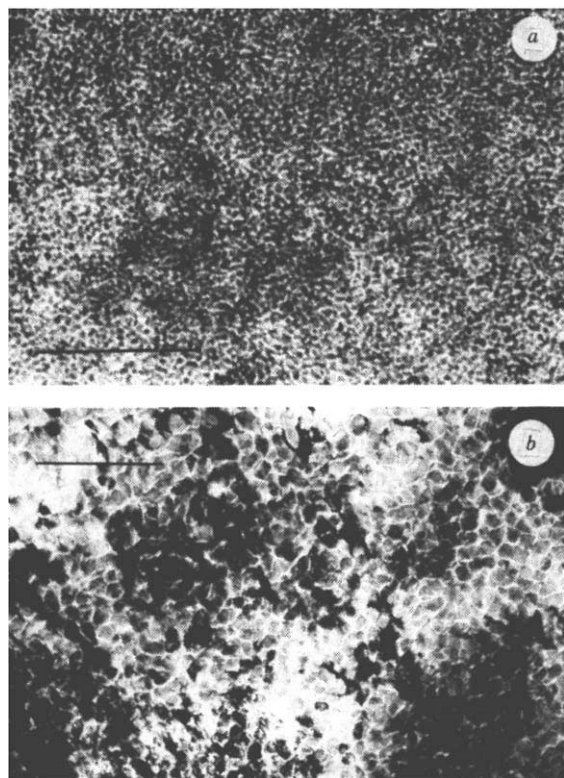


Fig. 1 Bright field transmission electron micrographs of cloudy taenite in *a*, Santa Catharina; *b*, Cape York. Scale bars: *a*, 0.2 μm ; *b*, 0.5 μm .

taenite and in the oxidised taenite phase first described by Lovering and Anderson⁵. The microstructure is very similar to that of cloudy taenite (Fig. 1*b*), but it lacks the usual crisp definition and the scale is very much finer. Two kinds of electron diffraction patterns are observed: the most frequent contains arcs corresponding to γ and α phases, but the other reveals the presence of two taenite orientations with only very weak reflections corresponding to kamacite. Figure 2 shows the latter type of diffraction pattern, in which the spots correspond to the (200) matrix orientation (γ_1) and the arcs to a second taenite phase (γ_2) containing a spread of orientations. This second taenite phase is related to the first by a rotation of 30–40° about the common [200] foil normal. Dark field images of regions as in Fig. 1*a* show that γ_1 comprises the cells and γ_2 is contained in the walls of the cellular microstructure. The two taenite phases thus consist of interpenetrating single orientations analogous to the α and γ phases of conventional cloudy taenite. The positions

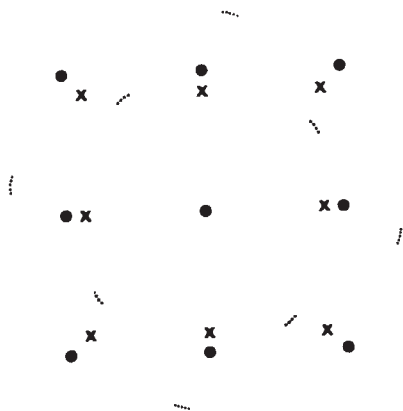


Fig. 2 Sketch to scale of electron diffraction pattern from Santa Catharina showing two taenite orientations and weak kamacite reflections (see text). $\bullet$, γ_1 ; $\times$, γ_2 ; $+$, α .

of kamacite reflections, which are very weak and would be lost in reproduction, are shown by crosses in Fig. 2. The orientation relationship between the kamacite and γ_1 is the Kurdjumow-Sachs relationship.

When a sample of the meteorite is heated for 1 h at 200 °C, slight general coarsening of the microstructure occurs, and the electron diffraction patterns show that arcs of diffracted intensity from the γ_2 phase disappear while the arcs of kamacite reflections are correspondingly strengthened. Annealing for 1 h at 400 °C produces considerable sharpening of these kamacite reflections, so that a recognisable duplex diffraction pattern (Fig. 3) results. This pattern contains extra reflections at positions corresponding to 110 γ and 100 α .

From their thermomagnetic analyses, Lovering and Parry⁶ suggested that Santa Catharina contained two taenite phases, as in the present case, with different Ni contents. Evidence for two γ phases (one ordered) in the taenite of octahedrites is also provided by the Mössbauer and electron diffraction studies of Knudsen *et al.*^{7,8}. The present result that one of the γ phases transforms into kamacite on heating was proposed by Tino *et al.*^{9,10} as transitional phases between γ -Fe and α -Fe, although these authors do not mention coexisting γ phases.

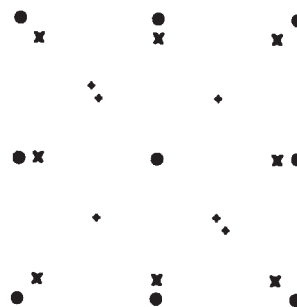


Fig. 3 Sketch to scale of electron diffraction pattern from Santa Catharina after annealing sample for one hour at 400 °C. $\bullet$, γ_1 ; $\times$, γ_2 ; $+$, extra reflections (see text).

Cloudy taenite has now been established as a duplex $\alpha + \gamma$ phase mixture by several workers^{1–3} in other iron meteorites. Bowles *et al.*¹¹ examined the two optically distinct (dark- and light-etching) regions of Santa Catharina and found that the dark matrix phase is taenite containing 30.7 at.% Ni, and claimed that the light phase is a dispersion of Fe_2NiO_4 in ordered taenite which contains 53 at.% Ni. The scale of these two taenite phases is several orders of magnitude greater than in the present work, where the fine duplex structure was found in both the light and dark regions. Although no evidence for the spinel was found, the extra γ reflections in Fig. 3 are consistent with ordered FeNi.

The morphology, fine particle size and single crystal nature of the phases in the duplex taenitic microstructure of Santa Catharina suggest its formation by spinodal decomposition of the parent taenite at low temperatures. A simple calculation shows that the fine scale of the microstructure is consistent with limited diffusion at quite low temperatures, such as 150 °C. The resulting structure is a 'precursor' cloudy taenite, one capable of modification to conventional cloudy taenite if heated to higher temperatures. The proposed reaction paths for the formation of cloudy taenite from the parent γ phase are thus: $\gamma \rightarrow \gamma_1 + \gamma_2 \rightarrow \gamma_1 + \alpha$. In other meteorites in which ($\alpha + \gamma$) cloudy taenite has been found, this reaction is believed to have already gone to completion, presumably spontaneously during cooling. Cases where two taenite phases are found to coexist thus represent an early stage in the decomposition of the metastable parent taenite. Further studies of Santa Catharina using chiefly Mössbauer spectroscopy are in progress.

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Indraloris and *Sivaladapis*: Miocene adapid primates from the Siwaliks of India and Pakistan

THE primate family Adapidae underwent a major radiation during the Eocene in Europe^{1,2} and North America^{3,4}. Asian and African Eocene mammalian faunas are still poorly known, but there is sufficient evidence to indicate at least a modest radiation of Eocene adapids in Asia^{5,6} and probably also in Africa². Apart from possible lemuriform and anthropoid primate derivatives, the family Adapidae was thought to have become extinct at the end of the Eocene (middle Tongrian, ~37 Myr (refs 2, 7, 8)). We present here new evidence which indicates that at least two genera of adapid primates, *Indraloris* and *Sivaladapis* (*gen. nov.*), survived into the late Miocene of India and Pakistan. These genera are little advanced over Eocene Adapidae in terms of dental adaptations and are apparently south Asian relicts of a much earlier radiation.

The history of species here placed in *Indraloris* and *Sivaladapis* is complex (Table 1). Pilgrim⁹ first proposed the genus *Sivanasua* in a footnote as a replacement for the preoccupied name *Ailuravus* Schlosser¹⁰. The type species of *Sivanasua* is thus the European procyonid carnivore *Ailuravus viverroides*. Pilgrim¹¹ later named two Asian species of *Sivanasua*: *S. palaeindica*, from Chinji beds near Chinji, Pakistan, and *S. himalayensis* from Nagri equivalent beds near Haritalyangar, India. Lewis¹² then named *Indraloris lulli* from Haritalyangar. Pilgrim regarded his Asian species of *Sivanasua*

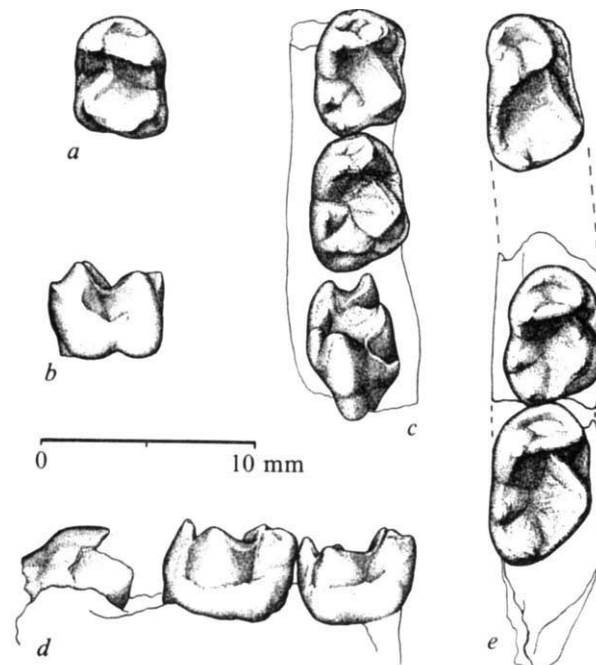


Fig. 1 Comparison of type specimens of *Indraloris* and *Sivaladapis*. *a*, *b*, *Indraloris lulli* (now placed in *I. himalayensis*), YPM 13802, left M_1 in occlusal and lateral view. *c*, *d*, *Sivaladapis nagrii*, GSI 18093, right mandible with M_{1-2} and impacted M_3 in occlusal and lateral view. *e*, *Sivaladapis palaeindicus*, GSI-D224, right P_4 and M_{2-3} in occlusal view. Note elongated molars with very large hypoconulids distinguishing *Sivaladapis* from *Indraloris*.

as carnivores, and Lewis regarded *Indraloris* as a lorissid primate “easily derived from the Adapidae”, but the specimens available to these authors were insufficient for unequivocal systematic placement. Tattersall¹³ later suggested that the type specimens of *S. himalayensis* (now lost) and *I. lulli* (Fig. 1*a*, *b*) are identical, and he regarded both as lorissid. Subsequently, *Indraloris* has usually been classified in Lorisidae^{14–16} or tentatively with Adapidae^{6,17}.

Sivanasua nagrii, described by Prasad¹⁸, is critical for understanding the systematic relationships of *Indraloris* and *Sivanasua*. The holotype of *S. nagrii* (Fig. 1*c*, *d*) is the first specimen to show unequivocally the presence of three molars in the lower dentition, ruling out any close relationship with procyonid carnivores such as European *Sivanasua* (Prasad orientated this specimen back to front and misidentified the impacted M_3 as P_4 , but further preparation confirms that three molars are present). *Sivanasua nagrii* is the only described specimen of Asian *Sivanasua* preserving the lower first molar (M_1), permitting the first direct comparison with the holotype of *I. lulli*. Judging from molar structure, species of *Indraloris* and Asian ‘*Sivanasua*’ are related, but they clearly represent two different genera.

No generic name is available for Asian species of ‘*Sivanasua*’. New specimens from Haritalyangar mentioned below indicate that these species are adapid primates, and we here propose the new name *Sivaladapis* to include the Haritalyangar species *S. nagrii* (Prasad) and the Chinji species *S. palaeindicus* (Pilgrim). The holotype of ‘*Sivanasua*’ *himalayensis* is probably a lower second molar (M_2) of the same species represented by the type specimen of *Indraloris lulli* (an M_1), and the correct name for this Haritalyangar species is thus *Indraloris himalayensis* (Pilgrim). The mandible with M_3 described by Tattersall¹³ is probably a specimen of *Sivaladapis palaeindicus*, as it comes from Chinji and has the morphology of this species.

Sivaladapis differs from *Indraloris* principally in having longer, relatively narrower lower molars, with a much larger hypoconulid (Fig. 1). The hypoconulid in *Sivaladapis* is twinned with the entoconid, and these two cusps are separated by a deep

Table 1 Named species of *Sivanasua* and *Indraloris* from the Miocene of India and Pakistan

Species	Type locality	Associated fauna ²² (European equivalent)
(1) ‘ <i>Sivanasua</i> ’ <i>palaeindica</i> Pilgrim, 1932 (now placed in <i>Sivaladapis</i>)	Chinji (Pakistan)	Chinji (Astaracian)
(2) ‘ <i>Sivanasua</i> ’ <i>himalayensis</i> Pilgrim, 1932 (now placed in <i>Indraloris</i>)	Haritalyangar (India)	Upper Nagri (Late Vallesian)
<i>Indraloris lulli</i> Lewis, 1933 (conspecific with <i>S. himalayensis</i>)	Haritalyangar (India)	Upper Nagri (Late Vallesian)
(3) ‘ <i>Sivanasua</i> ’ <i>nagrii</i> Prasad, 1970 (type species of <i>Sivaladapis</i>)	Haritalyangar (India)	Upper Nagri (Late Vallesian)

Valid species are numbered in the order in which they were described.

notch. The trigonids of *S. nagrii* are narrower than those in *I. himalayensis*, and the buccal cingulid is better developed. *Sivaladapis nagrii* also differs from *S. palaeindicus* in the latter two characteristics (Fig. 1). *Sivaladapis* and *Indraloris* are sufficiently similar that there is little doubt that both belong in the same family.

We are preparing a detailed description of the dentition of *Sivaladapis nagrii*, based on new specimens collected by S. Khare from the vicinity of Haritalyangar¹⁹, but the main points of interest are outlined here. *Sivaladapis* had an upper and lower dental formula of 2.1.3.3. The mandibular rami were solidly co-ossified at the symphysis early in life. The incisors are slightly procumbent, but have spatulate crowns and no indication of a dental scraper or tooth comb. This effectively rules out any close relationship to extant Asian lorises. The canines are relatively large and projecting, with a well developed dental hone on P₂ sharpening the back of the upper canine. The upper and lower fourth premolars are highly molarised. Upper molars have mesostyles and no hypocone. Average body size of *Sivaladapis nagrii*, estimated from tooth size²⁰, was about 5–6 kg. The highly crested crown morphology of the molars and molarised premolars, together with body size²¹, indicate that *Sivaladapis* was probably predominantly folivorous. *Indraloris*, by comparison, has less sharply crested molars and probably included relatively more fruit in its diet. A fused mandibular symphysis, spatulate incisors and projecting interlocking canine teeth honed by P₂ are adapid characteristics. *Sivaladapis* lacked the tooth comb characteristic of Lorisioidea and Lemuroidea. The cheek teeth of *Indraloris* and *Sivaladapis* resemble those of lorisoidea and lemuroid primates (such as *Hapalemur*) as well as most Adapidae, but the presence of a distinct hypoconulid twinned with the entoconid is found among lemuroid primates only in adapids such as *Hoanghoni* and *Oligopithecus*. Taken together, these dental characteristics leave little doubt that *Sivaladapis* and *Indraloris* belong to the family Adapidae.

Adapid primates are first known from the early Eocene. Inclusion of *Sivaladapis* and *Indraloris* in the Adapidae extends the latest record of this family from latest Eocene (~37 Myr (ref. 8)) to the late Miocene (~10 Myr (ref. 22)). This 27-Myr extension more than doubles the known stratigraphic range of the family. It also emphasises how little we know of the radiation of primates in Asia. Hyaenodontid creodonts were the dominant carnivorous mammals and adapids were the dominant primates in Eurasia during the Eocene. The last representatives of both these groups are now known from Siwalik faunas, and it is clear that the Indian subcontinent sheltered several archaic faunal elements as relicts through the Miocene.

The final extinction of Adapidae may have coincided with a major faunal turnover on the Indian subcontinent near the end of the Miocene (~8 Myr (ref. 22)). This faunal turnover included the first introduction of leaf-eating cercopithecoidea monkeys, identified as *Presbytis sivalensis*. Judging from their teeth, *Sivaladapis* and *Indraloris* may have been the ecological precursors of cercopithecoidea monkeys in south Asia, but more detailed stratigraphical and palaeoecological data in Siwalik faunas are required before any specific hypothesis can be advanced to explain the extinction of Adapidae. We now know that this important Tertiary primate family survived much longer than was previously thought.

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Cranial anatomy and implications of *Dolichocebus*, a late Oligocene ceboid primate

THE very scarce fossil record of Cainozoic New World monkeys^{1,2} has contributed little to knowledge of the history of platyrrhine primates, an important element of both the neotropical mammal fauna³ and the pantropical primates, as a whole. Only the affinities of the Middle Miocene Colombian fossils *Neosaimiri*, *Stirtonia* and *Cebupithecia* seem reasonably well established⁴, though not without dissent⁵, and these are clearly linked with the modern squirrel, howler and saki-uakari monkeys, respectively. After completion of a survey of the morphology and interrelationships of the platyrrhines, to be detailed elsewhere (A. L. R., in preparation), it is now possible to discuss the evolutionary implications of the terminal Oligocene *Dolichocebus gaimanensis* of Patagonia, represented by a nearly complete cranium only recently prepared fully, although first described in 1942 (ref. 6). This specimen strongly suggests that *Dolichocebus* is a member of the *Saimiri* lineage, which thus becomes the oldest generic lineage known for the primates, dating from about 25 Myr ago⁷. Its affinities also imply that the two major monophyletic divisions of Ceboidea were already established by late Oligocene times, as were the marmosets and tamarins.

A restoration of the *Dolichocebus* cranium is presented in Fig. 1. It is close in size to the modern middle-sized ceboids *Saimiri*, *Aotus* and *Callicebus* and the late Oligocene–early Miocene *Tremacebus* and *Homunculus*^{8,9}. During fossilisation all permeable cavities were filled with a fine sand that later hardened with the skull into a single mass. Most subsequent damage involved plastic deformation, bilaterally compressing the neurocranial vault asymmetrically above the skull base and partially collapsing the left orbit. The relatively undistorted basicranium affirms Kraglievich's⁹ nominal interpretation of the long, narrow skull. Because essentially all tooth crowns are missing and the posterior dental arch was shorn away bilaterally, the number of molars cannot be counted with certainty. However, the highly compact, alveolar-like texture of the exposed bone suggests that third molars were present in life⁸, contrary to Kraglievich's interpretation⁹.

There are several indications that the masticatory system of *Dolichocebus* was rather lightly built. The anterior and posterior roots of the right zygomatic arch, suggestive of the bending stress imposed by the attached masseter muscles, are quite gracile. Similarly, the pyramidal process of the palatine is slender, providing evidence that the pterygoids were not powerfully developed. The right temporal line is visible along much

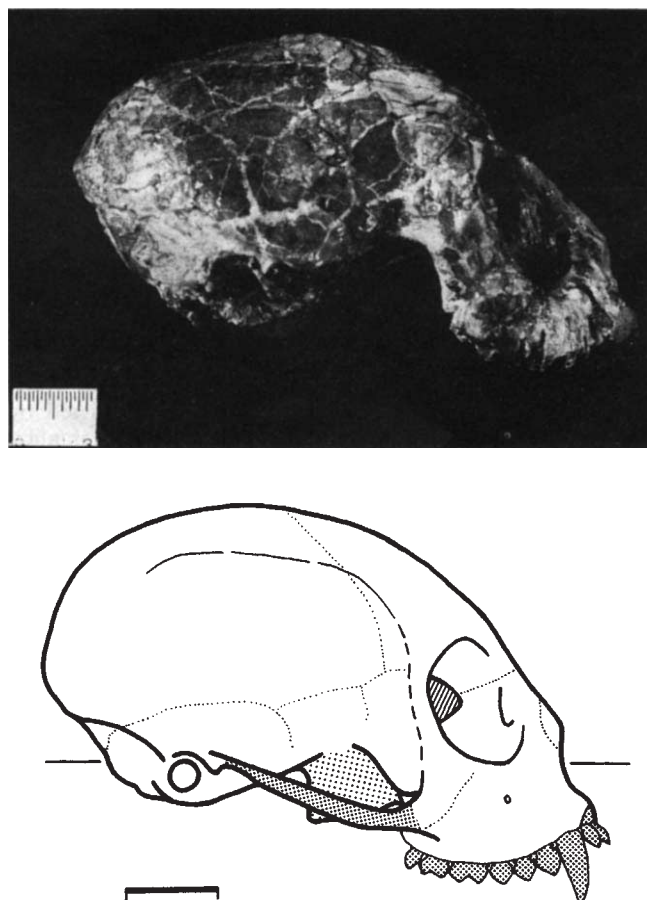


Fig. 1 *Dolichocebus gaimanensis*. Lateral view of original (top) and reconstruction (bottom). The scales are in mm and cm. Stippled areas are restored and hatchures mark the interorbital fenestra.

of the parietal near the midline and on the zygomatic wall of the temporal fossa. It is neither deeply etched nor rugose and the temporal fossa is transversely shallow, suggesting that the temporalis muscle was not well developed. Finally, the glenoid fossa is shallow and lacks an entoglenoid process medially, indicating that the compressive loading borne by the mandibular condyle did not have a strong mediolateral component. This might reflect a relatively small torque of the medial pterygoid-masseter muscular sling, which in turn implies an insertion on a relatively shallow mandibular corpus.

This pattern is very much like that of *Saimiri*. The gracile features of *Dolichocebus* and *Saimiri* contrast with the heavier homologous features of *Callicebus*, *Aotus* and *Pithecia*, for example, whose chewing muscles are correspondingly better developed¹⁰, and probably also of *Tremacebus* and *Homunculus*. The area of the neurocranium of *Dolichocebus* encompassed by the temporal muscles was relatively larger than that of *Saimiri*, however, especially posteriorly, where the fibres of the posterior temporalis converged on the midline and inion. In *Saimiri* the lines sweep ventrolaterally once they cross the coronal suture, delimiting a more limited area of insertion. These differences correlate with the relatively larger, hence more prognathous, face and enhanced dentition of the fossil. Given the limitations of the material, one can be sure only that the diet of *Dolichocebus* did not consist predominantly of leaves but of insects and fruit, perhaps as with *Saimiri* and/or *Cebus*^{11,12}.

The structure of the orbits establishes the phylogenetic connection of *Dolichocebus* and *Saimiri* to a very high degree of probability. In both taxa the juxtaposed and fused right and left medial orbital walls are perforated centrally by a relatively large

fenestra (in life closed by a sheet of connective tissue). Such a large interorbital fenestra is rare among extant adult mammals¹³, and is probably best developed in *Saimiri*. In a randomly selected series of 53 *Saimiri* skulls in the American Museum of Natural History (25 adult male, 17 adult female and 11 juvenile) from Columbia, Ecuador, Guyana and Peru, all individuals have a completely formed fenestra such as no other platyrrhines are reported to have¹⁴.

Although the functional significance of the interorbital fenestra is dubious, its unique structure and rare occurrence suggest that it is a (homologous) shared derived feature of *Dolichocebus* and *Saimiri*, illustrating that they are more closely related than either is to any other ceboid for which cranial material is available. (Specifically, this does not exclude the possibility that *Neosaimiri* or the Hispaniolan *Saimiri bernensis*¹⁵, known by dental remains only, are more recently related phylogenetically to *Saimiri*.) Other derived features which support *Dolichocebus-Saimiri* affinities are the great anteroposterior length of the frontal interorbital process, unique to these species, and the narrow nasals and reduced petromastoids, both indications of broader cebine affiliations.

Longstanding notions of the interrelationships of New World monkeys due to traditional classifications^{16,17}, or more modern ideas based on molecular evidence^{18,19}, are being challenged by cladistic analyses²⁰ and replaced by a new approach to platyrrhine classification²¹. It has been suggested, for example, that the reduced third molars and enlarged premolars of the cebines *Cebus* and *Saimiri* are indications that they are more closely related to the clawed marmosets (and tamarins) than to any other platyrrhine group with which they have been commonly classified. Furthermore, cebines and marmosets (Cebidae) form a monophyletic assemblage collaterally related to a second monophyletic amalgam composed of all remaining ceboids (Atelidae) with the single exception of the early Oligocene *Bransellia*. Some adaptive aspects of this dichotomy are marked by the contrast between light weight, frugivorous-insectivorous, and heavy, frugivorous-folivorous, masticatory systems.

Thus these larger lineages must have differentiated before the Colhuehuapian appearance of *Dolichocebus* in the late Oligocene, approximately 25 Myr ago⁷. This is supported by evidence of the relationships of the roughly contemporaneous *Tremacebus* and *Homunculus* to members of the alternative atelid clade. The living marmoset genera, which according to molecular evidence may have diversified only 7–10 Myr ago²², must also have differentiated before the late Oligocene. The historical details of this early radiation of the ancestors of modern ceboid primates and their extinct relatives requires the discovery of more fossils. Additional evidence may show that the diversity of living ceboids is a product of their early division into several long-lived, relatively anagenetic lineages, contrasting with the successional adaptive replacements revealed in the fossil record of Old World higher primates²¹.

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Evidence against a hydrodynamic function for fish schools

THERE are numerous explanations for the formation of fish schools^{1,2}, one of the most popular being that the members gain hydrodynamic advantage over solitary individuals^{3–6}. The model proposed by Weihs^{5,6} for the first time makes precise predictions about school structure which can be verified. We report here the first empirical test of this model, and demonstrate that three species of schooling fish do not swim in appropriate positions to gain hydrodynamic advantage.

Weihs considered three types of hydrodynamic advantage which could result in energy savings to fish (Fig. 1) and predicted the positions fish should take up to benefit from them (for details of the models see refs 5 and 6). We attempted to discover how well fish in schools took up these positions, as even approximations of the optimal spacings would provide significant advantage^{5,6}. To test the model, we made extended videotape records of the three-dimensional positions of fish in schools. The schools consisted of 20–30 saithe (*Pollachius virens*), herring (*Clupea harengus*) and cod (*Gadus morhua*), cruising in an annular channel 1.8 m wide and 31 m in circumference^{7–10}.

Weihs makes four mathematically convenient assumptions which we question for real schools. First, the effects require infinitely large schools, although schools more than 20 fish wide might approximate to this requirement^{5,6}. The schools which we filmed were much smaller, but probably contained two or three of the partially independent sub-units of which larger schools are composed⁷. Second, the model assumes all the fish are the same length, a condition rarely met in nature^{3,11}. Third, Weihs assumes that schools consist of several discrete layers, but this turns out not to be the case for saithe, cod, herring or minnows (*Phoxinus phoxinus*)^{7–10}. Finally, the model assumes that fish and their near neighbours swim on the same horizontal level, but fish tend to swim somewhat above or below their neighbours^{7–10,12}.

The existence of thrust vortices has been disputed, but we have demonstrated vortices in the positions predicted by Weihs (Fig. 2). Other predictions of the model, however, are not met. Although all three species definitely exhibited non-random spacing^{7–10}, none took up positions approximating those required by the model (Table 1A, B). Fishes' snouts were often in front of the tails of fish they were following⁷, and in these cases thrust vortices would not be produced in time to be used.

Fish had no tendency to position themselves centrally between pairs of fish swimming ahead of them, as would have been predicted had they been attempting to utilise their vortex trails (Table 1C). Furthermore, differences between observed and expected values were just as great for artificially culled schools in which all fish were the same length as in normal schools. Finally, we tested the prediction (see Fig. 1) that neighbouring fish should beat their tails in anti-phase. We found that neighbours showed no consistent phase relationship.

Although the species we tested clearly do not maintain positions predicted by the model, a fish which found itself in the 'correct' place to benefit hydrodynamically from its fellows might nonetheless be expected to remain there. In only 4 of 659 cases where an individual fish was found directly behind a pair of appropriately spaced neighbours was the fish both centred and far enough back to take advantage of the vortices.

Our fish could not have benefited from induced velocity from vortex trails. At first sight, however, our data do not rule out the possible effect between lateral neighbours. Observed values of about 0.9 body length separation are three times that predicted, but could still provide energy savings of 35% (refs 5, 6). This would only be the case, however, if fish swam on the same level as their neighbours, and usually they do not. We are left with the question of why natural selection has not favoured fish which take advantage of the fivefold energy savings predicted by Weihs. Part of the answer must lie in the model's implicit requirements for altruism. Fish in one row must take up specific positions so that other fish gain from their vortices^{13,14}. Also, only every other row of fish could benefit^{5,6}. Maximum possible benefits are calculated as the average for odd and even rows, but

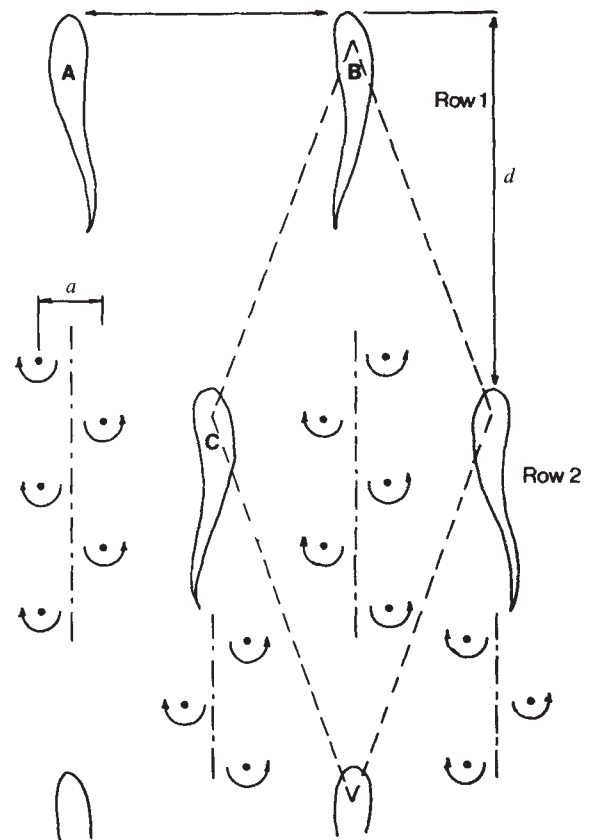


Fig. 1 Diagram of part of a layer of a hypothetical fish school. Dotted line indicates Weihs' diamond lattice. Fish swimming in these places gain fivefold energy savings due to three hydrodynamic effects: (1) fish C, mid-way between A and B, the pair of fish in front, would receive induced velocity from the spinning vortices shed by A and B. Thrust vortices do not become stable immediately, so fish C should stay more than 5 tailbeats behind the pair in front (d). Fish A and B should swim 0.4 body length apart (a), and beat their tails in anti-phase. (2) Fish swimming in close proximity can push off one another, thereby reducing the energy expended at a given swimming speed. Maximum savings occur at a separation of 0.3 body length. (3) Water flowing over extended pectoral fins might produce an updraft for the fish behind. This would be advantageous only for negatively buoyant fish, and so is not applicable to the fish studied. Also, the effect only occurs if the fins are held rigid and protrude beyond the maximum excursion of the tail, and this rarely seems to hold in nature.

Table 1 Predicted and observed spacing between fish

Species	Fish length Mean $\pm$ s.d. (cm)	Average cruising velocity (body length per/s)	Fish spacing					
			A lateral		B fore and aft		C centred	
			Predicted (cm)	Observed (cm (s.e.m.))	Predicted (cm)	Observed (cm (s.e.m.))	%	(n)
Saithe	27.1 $\pm$ 0.7	1.0	11.0	30.9(3.0)	96.3	70.0(7.4)	8	(24)
		1.3	11.0	34.5(2.6)	96.3	51.8(3.8)	29	(73)
		1.6	11.0	34.0(6.3)	96.3	58.1(4.5)	26	(140)
		1.9	11.0	32.1(2.7)	96.3	48.0(2.4)	25	(106)
		2.2	11.0	26.7(2.2)	96.3	40.7(1.5)	19	(194)
		2.5	11.0	29.6(3.0)	96.3	51.7(3.8)	22	(98)
Saithe	31.7 $\pm$ 5.2	1.4	12.3	33.8(4.6)	107.3	41.4(4.7)	33	(179)
		1.9	12.3	30.0(4.6)	107.3	38.6(3.3)	34	(186)
Herring	12.0	2.2	5.0	8.5(2.9)	43.8	11.9(2.6)	28	(74)
Cod	48.5 $\pm$ 5.5	1.9	16.0	30.5(3.9)	140.0	39.8(5.1)	23	(281)

Fish maintained mean distances from lateral neighbours 2–3 times as great as expected and swam far too close to pairs of fish in front of them when such pairs were present. Furthermore, although the model predicts no effects of swimming speed as the vortices remain in the same place, mean distance between fish is smaller at higher cruising speeds. The 31-m circular path which the fish swam might have slightly altered predicted positions of vortices, but, particularly for the 12-cm herring, this effect is probably smaller than disturbances due to turbulence expected in the wild.

selection should favour fish interested in staying in 'benefit' rows, so the situation is not evolutionarily stable¹⁵.

The lateral effect is quite different in this regard; individuals benefit from their own behaviour. If fish do not take up optimal positions for this advantage then other pressures must prevent them from doing so. For example, fish swimming close to their neighbours would be unable to turn quickly if the school was attacked. Also, swimming on the same level as close neighbours would restrict fishes' fields of view. Experiments investigating the sensory basis of schooling⁷ suggest that retaining manoeuvrability and visual range are more important than possible energy savings from the lateral effect.

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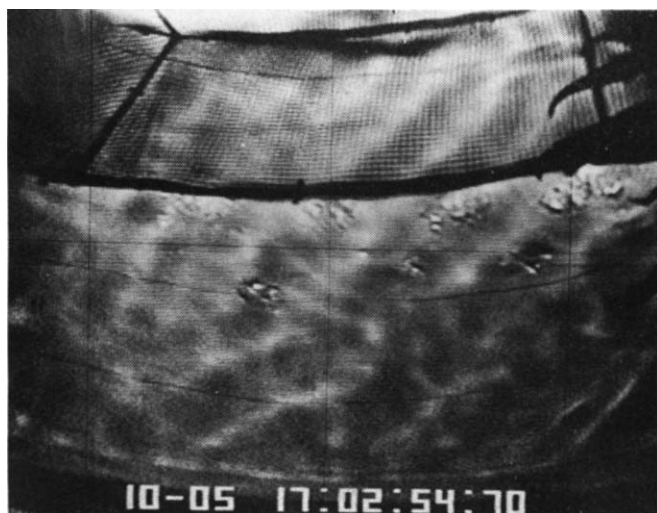


Fig. 2 Photograph from videotape showing stationary thrust vortices in positions predicted by Weihs. Distance between the vertical lines on the photo is about 1 m. Previous demonstrations of vortices^{16–18} have been criticised as resulting from boundary interactions. Our visualisation was produced by shining monochromatic light at a shallow angle to the water surface. Slight disturbances generated clear shadows on the tank bottom. Vortices in this photo were made by a fish swimming 20 cm below the surface. The vortices appear after a time proportional to the depth of the fish, proving that they are generated below the water surface.

An evolutionarily stable strategy approach to indiscriminate spite

AN individual behaves spitefully when it harms itself in order to harm another individual more¹. Hamilton^{1,2} predicted that spite may evolve if it is expressed only in those encounters that occur between individuals of less than average relatedness. More recently Verner³ suggested that territory size may become super-optimal because of a selective advantage arising from the spiteful exclusion of others from limited resources. His model is

Representation of possible outcomes			Probabilities		Total payoffs to players of		Expected fitnesses of strategies
			1 Patch 1 mutant	P-1 Patches no mutants	X_p	X_m	
First	Second	Third					
X_m	X_p	X_p	$\frac{1}{n}$	0	$f(2)+f(1)$	$f(3)$	$\bar{W}_{X_p} = \left[\left(\frac{f(2)+f(1)}{n} + \frac{f(2)+f(1)}{n} + \frac{2f(2)}{n} \right) + \frac{(n-6/2)3f(2)}{n} + (P-1)3f(2) \right] / (Pn-1)$
X_p	X_m	X_p	$\frac{1}{n}$	0	$f(2)+f(1)$	$f(3)$	
X_p	X_p	X_m	$\frac{1}{n}$	0	$2f(2)$	$f(2)$	$\bar{W}_{X_m} = \left[\frac{f(3)}{n} + \frac{f(3)}{n} + \frac{f(2)}{n} \right] / 1$
X_p	X_p	X_p	$\frac{n-(6/2)}{n}$	1	$3f(2)$	0	

Fig. 1 The possible outcomes when individuals settle out at random from a pool of n competitors to fill patches with 6 resource units. In one patch competitors consist of $n-1$ individuals who play $X_p=2$, and a single mutant individual who plays $X_m=3$. In the remaining $P-1$ patches all competitors play the strategy X_p . A particular individual, say the mutant in one patch, may settle first, second, third or not at all. Note that the last individual to settle takes either the number of resource units dictated by its strategy or the number left in the patch, whichever is smaller. The mean expected fitness of an individual playing a particular strategy is equal to the total expected payoffs to all individuals playing the strategy divided by the number of individuals playing the strategy.

essentially different from Hamilton's in that spite is directed at individuals indiscriminately with respect to relatedness. Recently Rothstein⁴ has shown analytically that the initial spread of spiteful traits will be very slow in all but the smallest populations. He also argued verbally that indiscriminate spite can never be evolutionarily stable even if it should spread (see also Davies⁵). The question of evolutionary stability is clearly important, but its resolution requires an analytical approach. We report here an approach based on Maynard Smith's⁶ concept of the evolutionarily stable strategy (ESS), a strategy which, when common, does better than any alternative strategy played by a rare mutant. We show that spite can be an ESS, but that the magnitude of spite will be small in large populations.

Consider a territorial species with non-overlapping generations in which individuals compete for a finite number of resource units essential for reproduction. The resource units are distributed in patches, each of which has a pool of competitors for the resource units. At each patch individuals settle at random, one by one, out of the pool until no more resource units are available.

Let: R = the number of resource units in a single patch; P = the number of patches; X = the number of resource units defended by an individual; X_p = the strategy for resource use (the number of resource units defended) established in the population; X_m = the strategy played by a single mutant in one of the P pools of competitors; $f(X)$ = the fitness of an individual defending X resource units ($0 \leq f(X) \leq 1$), expressed as the product of gross benefit ($b(X)$) and cost ($c(X)$); X_{opt} = the strategy which maximises individual fitness ($b(X)c(X)$); V = a constant that converts $f(X)$ into the number of surviving

offspring produced by an individual; $n(X_p)$ = the number of individuals in generation $t+1$ competing for the R resource units of each patch when the population strategy of generation t was X_p ; we assume here that $n(X_p) = f(X_p)VR/X_p$; $\bar{W}_X$ = the mean fitness of individuals playing strategy X .

We wish to find a value for the strategy X_p such that the mean expected fitness of individuals playing X_p ($\bar{W}_{X_p}$) exceeds the expected fitness of a mutant individual playing any alternative strategy X_m ($\bar{W}_{X_m}$). Figure 1 shows how $\bar{W}_{X_p}$ and $\bar{W}_{X_m}$ can be precisely calculated for specific cases. Unfortunately, although general formulations for $\bar{W}_{X_p}$ and $\bar{W}_{X_m}$ can be obtained in terms of R , X_p and X_m , they are mathematically intractable. For this reason we have used the following approximations (written in a form parallel to the precise examples in Fig. 1):

$$\bar{W}_{X_p} = \left[\underbrace{\left(\frac{R/X_p}{n(X_p)} \right) \left(\frac{R-X_m}{X_p} \right) f(X_p)}_{\text{1 patch, mutant gets in}} + \underbrace{\left(\frac{n(X_p)-R/X_p}{n(X_p)} \right) \left(\frac{R}{X_p} \right) f(X_p)}_{\text{1 patch, mutant does not get in}} + \underbrace{(P-1) \left(\frac{R}{X_p} \right) f(X_p)}_{\text{P-1 patches, no mutant}} \right] / \underbrace{(Pn(X_p)-1)}_{\text{No. individuals playing } X_p} \quad (1)$$

$$\bar{W}_{X_m} = \left(\frac{R/X_p}{n(X_p)} \right) f(X_m) / \underbrace{1}_{\text{No. individuals playing } X_m} \quad (2)$$

(assuming $n(X_p) \geq R/X_p$, $n > 1$, $R \geq X_p$, $R \geq X_m$, prob. = probability).

We now solve for the ESS, using the technique described by Parker and Macnair⁷, as follows. If X_{ESS} is the ESS for the number of resource units taken, then it must be more fit when common than any single mutant which takes $X_{ESS}Y$ resource units ($Y > 0$). Thus if we subtract the expected fitness of a single mutant taking $X_{ESS}Y$ units from the mean expected fitness of individuals taking X_{ESS} units, the result is positive except when $Y = 1$, when the result is zero.

$$\therefore \text{ when } Y = 1, \left. \frac{d}{dX_{ESS}Y} [\bar{W}_{X_p=X_{ESS}} - \bar{W}_{X_m=X_{ESS}Y}] \right|_{X_{ESS}} = 0 \quad (3)$$

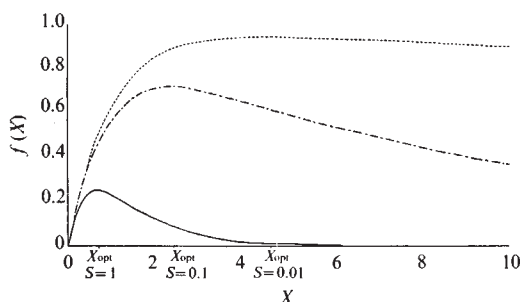


Fig. 2 The relationship between the number of resource units defended and individual fitness for three fitness functions. X_{opt} is the value of X which maximises the number of surviving offspring produced ($f'(X_{opt})=0$). Solid line, $S=1$; dash-dot line, $S=0.1$; dotted line, $S=0.01$. $f(X) = b(X)c(X) = (1 - \exp(-SX)) \exp(-SX)$.

Substituting equations (1) and (2) into equation (3) gives: when $Y = 1$,

$$\frac{d}{dX_{ESS}Y} \left[\left[\left(\frac{R/X_{ESS}}{n(X_{ESS})} \right) \left(\frac{R - X_{ESS}Y}{X_{ESS}} \right) f(X_{ESS}) + \left(\frac{n(X_{ESS}) - R/X_{ESS}}{n(X_{ESS})} \right) \left(\frac{R}{X_{ESS}} \right) f(X_{ESS}) + (P-1) \left(\frac{R}{X_{ESS}} \right) f(X_{ESS}) \right] / (Pn(X_{ESS}) - 1) - \left(\frac{R/X_{ESS}}{n(X_{ESS})} \right) f(X_{ESS}Y) \right] \Big|_{X_{ESS}} = 0 \quad (4)$$

Differentiating, holding X_{ESS} constant, and then substituting $Y = 1$ eventually resolves to:

$$X_{ESS} = \frac{-f(X_{ESS})}{f'(X_{ESS})(Pn(X_{ESS}) - 1)} \quad \text{or} \quad f(X_{ESS}) \left[PVR + \frac{1}{f'(X_{ESS})} \right] \quad (5)$$

(as we assume that $n(X_{ESS}) = f(X_{ESS})VR/X_{ESS}$).

The same technique may also be used to determine whether X_{ESS} can invade any population playing $X_{ESS}Y$ (an ESS unable to do so is less likely to be found in nature as its ability to become established is more dependent on the starting conditions). In this case when $Y = 1$,

$$\frac{d}{dX_{ESS}Y} [\bar{W}_{X_p=X_{ESS}Y} - \bar{W}_{X_m=X_{ESS}}] \Big|_{X_{ESS}} = 0 \quad (6)$$

This also gives equation (5) as a solution, indicating that the ESS can invade a population playing an alternative strategy.

Examination of equation (5) and Figs 2 and 3 helps in understanding the biological meaning of these results. First, equation (5) shows that in populations of finite size X_{ESS} must be greater than X_{opt} (where $f'(X) = 0$) because only when $f'(X_{ESS})$ is negative can X_{ESS} be positive and thus biologically meaningful. Second, note that when the cost of spite is lowered (that is, $f(X)$ is increased and $|f'(X)|$ is decreased for values of X above the optimum; dotted lines, Fig. 2), the ESS level of spite is increased, as expected (Fig. 3). Third, as the number of competing individuals per patch $n(X_{ESS}) = f(X_{ESS})VR/X_{ESS}$ or the number of patches (P) increases (that is, as total potential population size increases), the ESS approaches the 'optimal' level, because $f'(X_{ESS})$ must get closer to zero as $Pn(X_{ESS}) - 1$ increases if the equality in equation (5) is to remain satisfied. Thus in populations of infinite size spite will never evolve (Fig. 3).

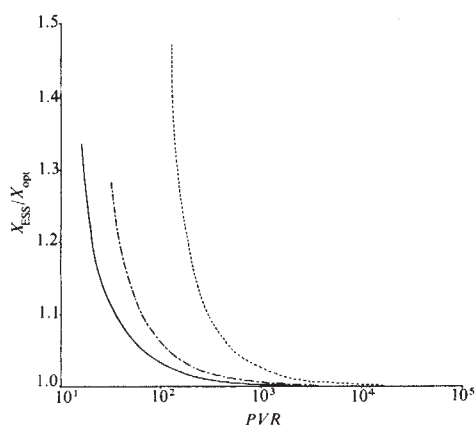


Fig. 3 The degree of spite, expressed as a ratio of the ESS number of resource units defended to the 'optimal' number, as a function of the cost of spite (as modified by the shape constant, S) and potential population size (PVR) using the same fitness functions as in Fig. 2. For each fitness curve, the ESS is equal to R (that is, 'take the entire patch') whenever PVR is below the range of values for which the ratio is plotted. Solid line, $S = 1$; dash-dot line, $S = 0.1$; dotted line, $S = 0.01$.

The ESS model presented above is only an approximation. First, it makes the following assumptions: (1) Any rare mutant which secures a place in a patch always takes the full number of resource units dictated by the strategy it is playing (that is, it is never forced to take less, as in the third outcome of Fig. 1). (2) The last X_p -playing individual to enter a patch, if forced to take some fraction α of X_p , has a fitness of $\alpha f(X_p)$ (a value which may be higher or lower than the true fitness $f(\alpha X_p)$). We compared some of the results presented in Fig. 3 with those obtained using iterations based on precise formulations which do not make these assumptions, however, and found that the differences between the two were generally less than 1% (manuscript in preparation). When the discrepancies were larger, then the approximate solutions were usually a conservative estimate of the degree of spite which can be evolutionarily stable. Second, there are no explicit genetic constraints on the models we have presented for the stability and spread of the ESS. We used the technique of Parker and Macnair⁷ to show that our results are unaffected by assumptions of dominance or recessiveness in single locus diploid models (unpublished results). No attempt has been made to model other types of genetic control, however.

In conclusion, we have provided an ESS model for the evolution of spite which considers a continuous strategy set in populations of finite size. Like Rothstein⁴, we found that highly spiteful behaviour is generally unlikely to occur, except in small populations, but our models contrast with his in indicating that some degree of indiscriminate spite can be an evolutionarily stable strategy.

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Parasitic, amoeboid dinoflagellates

SEXUAL reproduction has so far been unknown among the Dinococcales, unicellular brown algae belonging to the Dinophyceae (dinoflagellates). As we report here, however, the life cycles of two members of this group, *Stylodinium sphaera* and *Cystodinedria inermis*, include a parasitic amoeboid stage which brings about vegetative reproduction and also possibly sexual reproduction. We believe that the two amoeboid forms have previously been identified mistakenly as a separate taxon of protozoan.

Stylodinium sphaera Pascher and *Cystodinedria inermis* Geitler were abundant in an algal sample collected from Hubenov Dam Reservoir in Bohemia in July 1978 and taken to the Czechoslovakian Academy of Science Hydrobiological

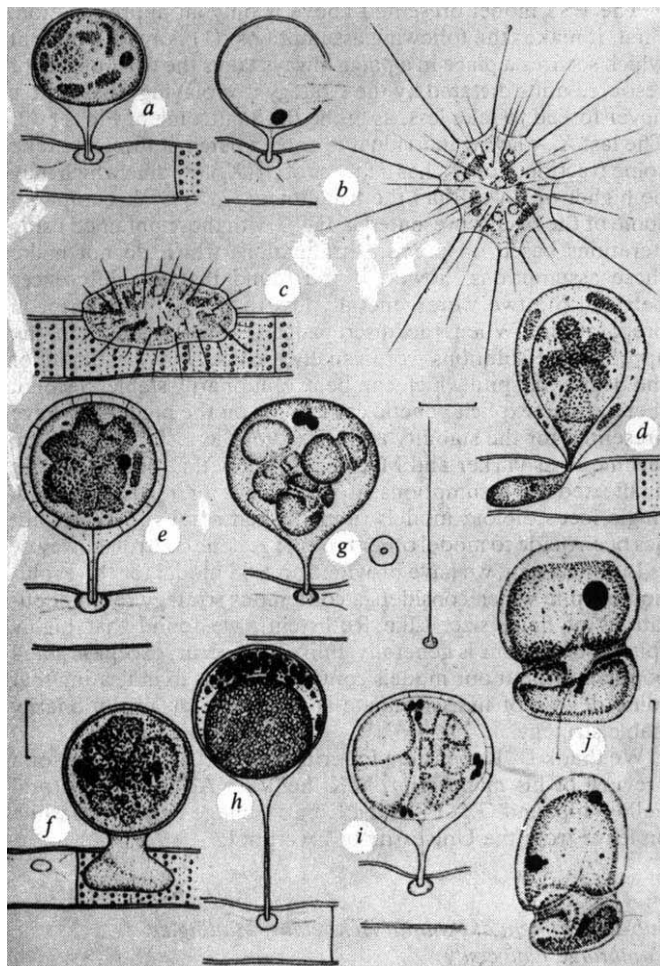


Fig. 1 *Stylodinium sphaera* Geitler. *a*, *Stylodinium sphaera*; *b*, empty *Stylodinium* cell and one of two amoebae which emerged from it; *c*, amoeba settled on *Oedogonium*; *d*, amoeba parasitising *Oedogonium* and starting to develop typical *Stylodinium* form; *e*, *Stylodinium* form complete; *f*, *Stylodinium* amoeba moving to parasitise second cell before developing non-motile form; *g*, formation of four or more zoospores or microspores (cross-section of stipe and single suctorian-like tentacle enlarged); *h*, *Stylodinium* cell with spore; *i*, formation of from large gymnodinoid-like cells; *j*, gymnodinoid-like cells—one slightly larger than the other—which are probable gametes.

Laboratory in Prague for further study. *C. setosa* Pascher, *C. maxima* Popovský and *C. aeruginea* Pascher were also present. They were epiphytic on *Oedogonium* and *Spirogyra* filaments.

Stylodinium (Fig. 1) has been described¹ as stipulate with cells globose to ovoid or quadrate. The stipe is said to be gelatinous, varying in length from less than half to more than double the cell diameter (Fig. 1*a*). According to the literature, the protoplast contains numerous parietal, discoid to elliptical, golden-brown chromatophores and one or more conspicuous globules of reddish oil¹. Thompson⁴ observed *Stylodinium* growing epiphytically on *Oedogonium* and reported its reproduction by division of the protoplast into two gymnodinoid zoospores liberated by rupture of the parent cell wall. We always observed *S. sphaera* attached to empty *Oedogonium* cells. Its size ranged from 25.5 to 31.7 $\mu\text{m} \times 15.0$ to 28.7 μm excluding the stipe. Stipes were one-third to one-and-a-quarter times as long as the cell diameter. Colour varied from bright green to brown to bright orange. All cells had one or more reddish globules. We saw many orange cells rupturing and liberating two orange amoebae (Fig. 1*b*), leaving behind remnants of the globules. Amoebae enlarged slightly as they emerged, possessed two flagellar-like pseudopodia, 8–12 contractile vacuoles and many fine pseudopodia in the form of axopods two to three times the

length of the cell. One axopod always ended in a disk-like structure. No nucleus was visible. Axopods shortened within seconds as an amoeba settled on an intact *Oedogonium* cell (Fig. 1*c*). A few seconds later it punctured the wall and swelled suddenly (10–15 s) as the *Oedogonium* cell was emptied of its contents (Figs 1*d*, 4*a*). On several occasions an amoeba parasitised two *Oedogonium* cells before developing the non-motile *Stylodinium* form with stipe (Fig. 1*f*). This accounts for the diversity in cell size observed in our sample. The more *Oedogonium* cells parasitised, the larger the typical non-motile *Stylodinium* cell.

When an amoeba ceased to be parasitic it changed into a typical non-motile brown cell within 30 min. Within a further 10 min the protoplasm of some cells divided into four to eight small gymnodinoid-like zoospores or microspores similar to those described for *Cystodinium*⁵. Other cells either formed four larger gymnodinoid cells with stigmas which may have been gametes, or turned orange and released amoebae. The protoplast of some cells formed a yellowish-brown spore with reddish globules above (Fig. 1*j*). Reddish globules present in all vegetative cells and left behind by emerging amoebae were probably undigested remnants of *Oedogonium*. Further, on at least one occasion, several pairs of the so-called *Stylodinium* zoospores were seen dancing around each other. These gymnodinoid-like

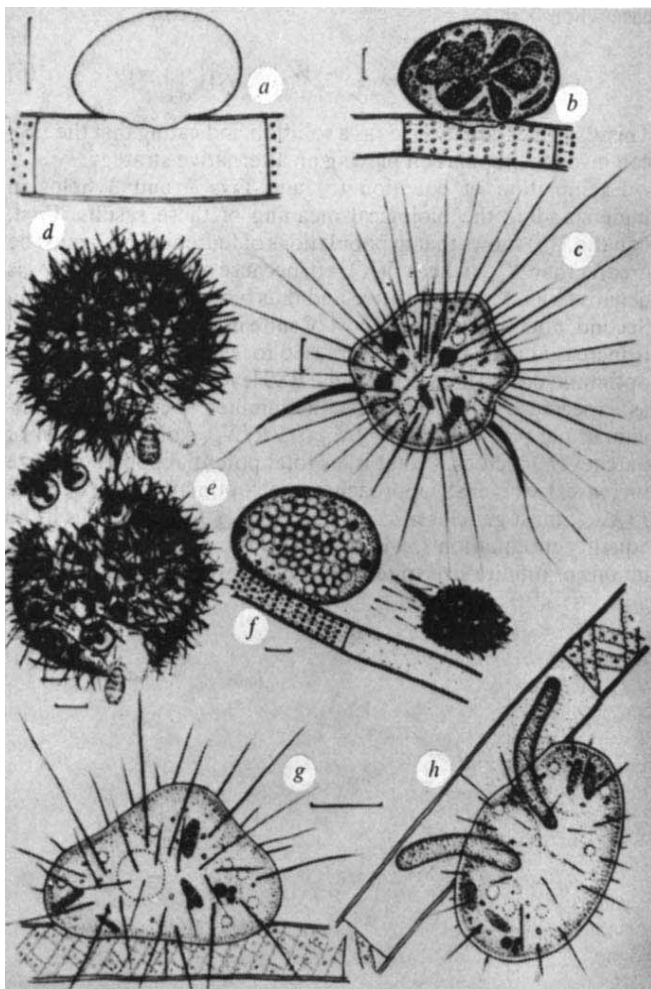


Fig. 2 *Cystodinedria inermis* Geitler. *a*, Empty *Cystodinedria* cell (25.0 $\times$ 17.0 μm) on *Oedogonium*; *b*, *Cystodinedria* after parasitising four cells—cell size is that of *C. maxima*; *c*, one of two amoebae liberated from large *Cystodinedria* shown in *a*; *d*, shelled *Cystodinedria* amoeba engulfing *Oedogonium* zoospore; *e*, shelled amoeba releasing microspore; *f*, *Cystodinedria* formed as result of microspores parasitising *Oedogonium*, with shell surrounding microspores discarded to right; *g*, one of two amoebae (15 to 17.5 μm in diameter) settled on *Spirogyra*; *h*, amoeba lyses *Spirogyra* wall and engulfs protoplast: cell resembles *C. setosa* at this stage.

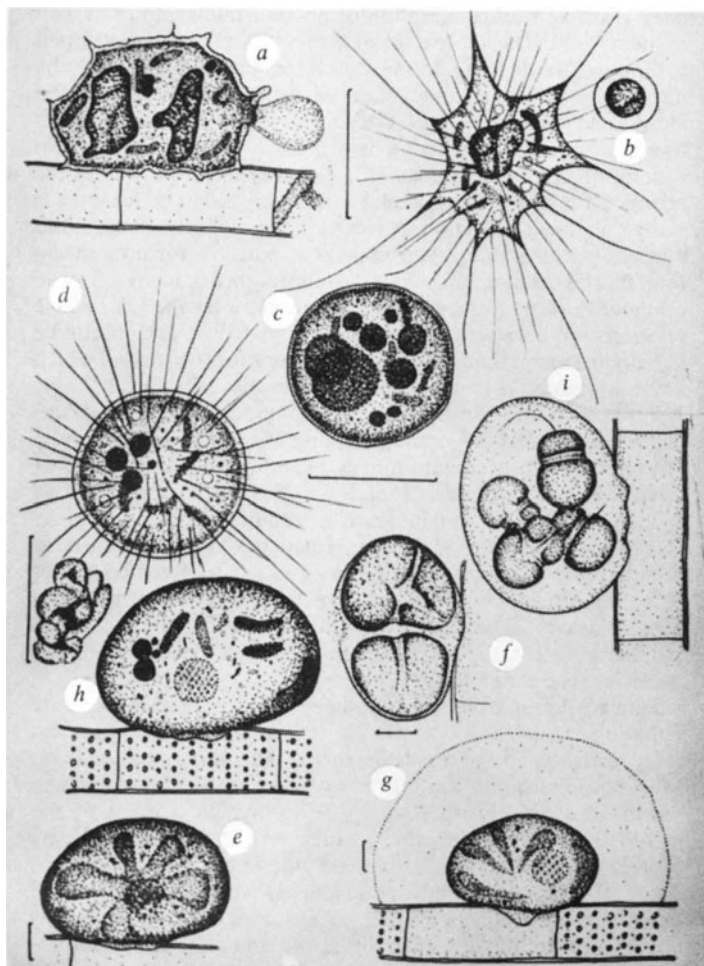


Fig. 3 *Cystodinedria inermis*. a, Pseudopodium withdrawn from *Spirogyra*; axopods not visible; b, amoeba leaves filament with flagella not visible; greenish spore fuses with amoeba; c, protoplasm divides into four to six spheres: shape metabolic; d, axopods and contractile vacuoles reform: cell content yellowish-orange; e, *Cystodinedria maxima* on *Oedogonium*; f, division of cell content into zoospores with stigma and flagella; g, zoospore becomes non-motile, surrounded by mucilage; h, green *Cystodinedria* cell, possibly starting to parasitise new cell; i, protoplast divided into gymnodinoid cells which are probable gametes. (e-i are taken from ref. 13.)

zoospores had a definite nucleus, two yellowish-brown chromatophores, a stigma and one or two reddish spheres. The transverse sulcus twisted left, the longitudinal one reached to the antapex. One member of the pair (Fig. 1) was slightly larger ($17.5 \times 16.0 \mu\text{m}$). We believe they may have been gametes because their behaviour was similar to that of other dinoflagellate gametes⁶⁻¹⁰.

A somewhat similar, though more complex life history of *Cystodinedria inermis* (Figs 2, 3, 4b) was observed. *Cystodinedria* (Fig. 2a) has been described as ellipsoidal with discoid chloroplasts, occurring as an epiphyte on filamentous algae^{11,12}. Reproduction (Fig. 3f) is said to be by zoospores^{3,13}. Our cells varied within the range 22.0 to $57.0 \mu\text{m} \times 17.0$ to $47.5 \mu\text{m}$. Colour varied from bright green to brown to orange. Each cell contained one to several reddish globules. Orange cells liberated two amoebae similar to those formed by *Stylodinium*. These amoebae moved across *Oedogonium* cells to *Spirogyra*. Axopods shortened and, 5 min after an amoeba had settled on a *Spirogyra* cell (Fig. 2g), the host's chloroplast uncoiled. Within 10 min the entire *Spirogyra* protoplast was consumed by the amoeba (Fig. 2h). The amoeba then moved to the next cell and began the process again (Fig. 3a). One amoeba parasitised four cells in a row before it rested on an intact *Spirogyra* cell. Some extended axopods and moved to new filaments (Fig. 3b). One

engulfed or possibly fused with what seemed to be a microspore. Another amoeba divided into four to six spheres after it left *Spirogyra*. After parasitising one to four *Spirogyra* cells amoebae became non-motile and resembled green *Cystodinedria* cells. Their colour changed to brown and within 2 h a gelatinous matrix formed around the cells. Eight hours later the cells left the filaments as shelled amoebae (Fig. 2d).

Shelled amoebae moved so slowly that they at first seemed to be motionless. Many such amoebae were present in the collection several days after our first observations. All had dead *Oedogonium* zoospores nearby. We saw *Oedogonium* zoospores approach shelled amoebae, become motionless, and become engulfed slowly (2 h). As *Oedogonium* zoospores were engulfed, the shell on the opposite side broke, and a small piece of shell and protoplasm (possibly in the form of microspores) left and quickly moved to *Oedogonium* (Fig. 2e). The shell was cast off as the small ($5-10 \mu\text{m}$) amoeba parasitised *Oedogonium* (Fig. 2f). After parasitising one to several *Oedogonium* cells, amoebae became motionless and assumed the typical *Cystodinedria* form^{3,11}. Gymnodinoid-like zoospores (Fig. 3f and i) reported and observed by J.P.¹³ were not seen in the

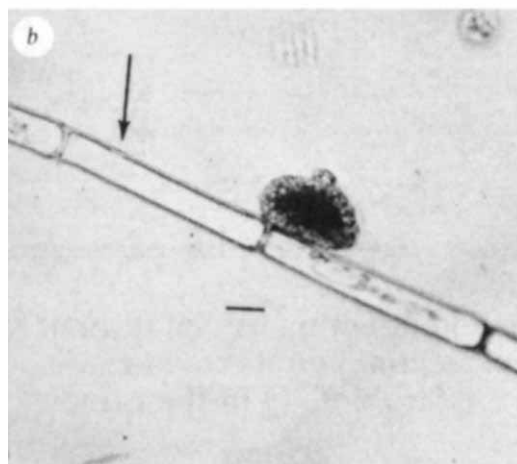
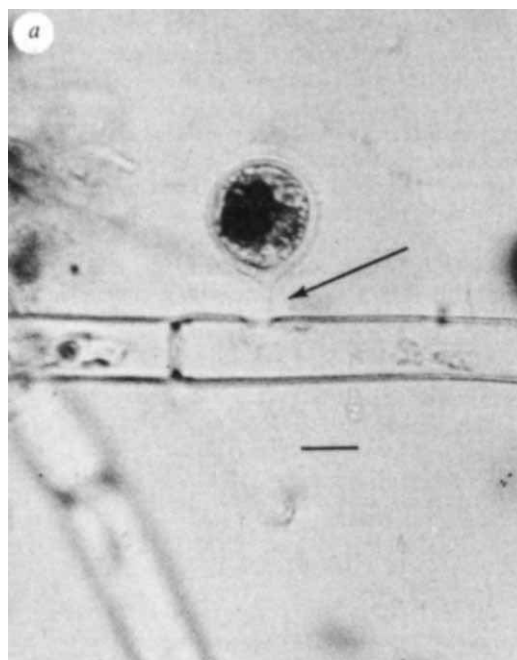


Fig. 4 a, *Stylodinium* attached to empty *Oedogonium* cell which it parasitised. The arrow points to the stipe. A reddish-brown globule is visible ($\times 660$). b, *Cystodinedria* parasitising second *Oedogonium* cell in a row. The arrow points to a hole made as it parasitised the first cell ($\times 400$).

Hubenov material. Hubenov¹³ observed one zoospore settle on *Oedogonium*, form a mucilage around itself, lose the girdle and sulcus and assume the typical *Cystodinedria* form. It is possible, however, that some of these motile cells act as gametes rather than zoospores. L.P. has observed fusion of such zoospores in *Cystodinium*, another member of the Dinococcales. Thus, sexual reproduction does occur in the Dinococcales. She has also observed parasitic amoeboid stages in that genus.

That the life histories reported here for *Stylodinium* and *Cystodinedria* are not aberrant is verified by the fact that J.P. collected these genera in Yugoslavia and Greece in August 1978 and L.P. collected them in August 1978 in Oklahoma. Both observed the same life histories reported here.

Parasitic, amoeboid stages reported here in the life history of *Stylodinium* and *Cystodinedria* have important systematic implications. Amoebae possessed one or two orange chromatophores. Chromatophores previously described for these genera are those of the algae which they parasitised. *Peridinium foliaceum* and *P. balticum* both contain a chrysophycean endosymbiont which is thought to control the development of their chloroplasts¹⁵. However, these chloroplasts are functional while those of *Stylodinium* and *Cystodinedria* are not. Amoebae observed in our study are very similar in appearance to several named algal and protozoan genera such as *Rhizochrysis* Pascher and *Vampyrella* Cienkowski. These genera may be a phase in the life history of other dinococcal life histories and thus invalid taxa. Amoeboid members of the Dinococcales should be moved to the order Dinamoebidinales which is presently monotypic. This study indicates that earlier descriptions of vegetative reproduction are invalid and suggests the need for systematic revisions in both protozoan and algal taxonomy.

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An independent microbial flora of the epithelium and its role in the ecomicrobiology of the rumen

It has been suggested that the bacterial flora of the rumen should be considered as three distinct, interacting populations—the bacteria of rumen fluid (the population which has been studied most extensively), the bacteria associated with food particles, and the bacteria adhering to the epithelial wall of the organ¹. Until now, studies of the 'epithelial' population have

been restricted to examination of postmortem samples of wall tissue and its attached bacterial flora^{2–5}. A recently developed technique⁶ for feeding young sheep for long periods solely by infusion of protein and other essential nutrients into the abomasum, and of volatile fatty acids and bicarbonate buffer into the rumen, has provided us with an opportunity to study in isolation the role of the bacterial population of the wall in the ecomicrobiology of the rumen in the living animal. Our studies show that this population can exist independently of the other two populations, that it is primarily responsible for urea digestion in the rumen and that it initiates breakdown of dead epithelial tissue. Furthermore, our results point to an inverse relationship between ammonia concentration and ureolytic activity in rumen fluid, which may account for the control which ammonia exerts over flux of urea across the rumen wall^{7–9}.

Four castrated Suffolk × (Finnish landrace × Dorset horn) lambs, aged 6–9 months and weighing 35–40 kg were surgically prepared and infused with nutrient solutions by the method of Ørskov *et al.*⁶ Casein was supplied to the abomasum at a level estimated to be 1.5 × maintenance. Volatile fatty acid solution (1.2 M acetic acid, 0.5 M propionic acid, 0.3 M butyric acid) was pumped into the rumen so that the animals received energy at twice maintenance. This solution was free from bacterial contamination. The sheep were fed solely by infusion for 2 months before samples of rumen fluid and excised epithelial papillae were examined.

Rumen liquor from infused sheep was in appearance quite unlike the thick fluid of normally-fed animals. It contained no food particles and was much less turbid than normal rumen fluid. Most of the turbidity was due to a white, spongy material which sedimented quickly on standing. The protein content of the mixed fluid was 0.3 mg ml⁻¹, much less than the 10–15 mg protein per ml of normal rumen fluid. The pH (6.6–7.0) and range of NH₃ concentrations (Table 1) were normal, but the oxidation–reduction potential, an *E_h* of –14 to –50 mV, was much closer to that of rumen fluid from uninoculated gnotobiotic animals (0 mV; R. J. W. and K.-J. C., unpublished) than to normal fluid, which is usually –250 to –450 mV (ref. 10). The concentration of lactate was <0.1 mM and urea was absent.

Viable counts of bacteria in rumen fluid from infused sheep showed that the lower cell density was due entirely to a reduction in the anaerobic count from >10⁹ to ~10⁷ ml⁻¹ or fewer, while the aerobic count actually increased (Table 1). Of 59 isolates from the highest dilution (10⁵ and 10⁶) anaerobic roll tubes, 48 (81%) also grew aerobically, showing that the fluid population was largely facultative. It may be assumed that the lack of carbohydrate substrate and the relatively high *E_h* prevented growth of many of the strict anaerobes typical of the normal rumen. Despite this quite abnormal flora, rumen liquor from infused sheep possessed urease activity within the range normally found in the rumen (Table 1; refs 1, 5, 11–15). Removal of bacteria by centrifugation (35,000g, 30 min) removed all urease activity from the fluid. Gentle centrifugation (100g, 5 min) removed the suspended white solid and at the same time removed about 50% of all urease activity from the fluid. Isolates from rumen liquor of infusion sheep were screened for urease activity, and of 113 isolates, 16 were ureolytic (Table 1). This proportion is very high compared with those found by Cook (6 from 1,200 isolates)¹¹ and others^{13,15}. However, the increased proportion is due mainly to the reduction in the numbers of strict anaerobes, and the ureolytic bacteria are again found in 10⁶ and 10⁵ dilutions of rumen fluid.

Papillae excised from the rumen wall of infused sheep had a similar appearance to papillae excised from the rumen wall of a normally fed sheep, except that they were slightly larger. Microscopy did not reveal any structural difference between the two. Some abrasion is necessary to maintain a healthy rumen wall, and in these sheep fed by infusion, plastic pan scrubbers were placed in the rumen to provide abrasion⁶. Counts of bacteria associated with the rumen wall were done by the same methods used for rumen fluid samples. Papillae were excised, washed in anaerobic culture medium and homogenised (10–

Table 1 Bacterial counts, ammonia concentration and urease activity in the rumen of sheep fed by intra-ruminal and intra-abomasal infusion and by normal feeding

Sheep	Diet	10 ⁻⁷ × no. of bacteria per ml rumen fluid		Ureolytic bacteria total isolates from rumen fluid		NH ₃ (mM)	Urease activity	
		Aerobic	Anaerobic	Aerobic	Anaerobic		Fluid	Epithelium
2938	Infusion	0.5 (3)	0.8 (4)	1/19	8/21	12.6 (3)	0.59 (3)	1.3 (2)
2975	Infusion	1.0 (1)	5.0 (1)	1/7	3/8	9.3 (3)	0.63 (3)	ND
2977	Infusion	1.1 (2)	0.9 (2)	1/8	0/12	8.9 (2)	0.64 (2)	1.0 (1)
3009	Infusion	0.6 (2)	0.6 (3)	0/19	2/19	4.5 (3)	1.54 (3)	4.4 (2)
2705	Hay	0.04 (3)	2.3 × 10 ² (3)	3/8	0/10	2.6 (3)	3.20 (3)	3.3 (2)

Serial tenfold dilutions of rumen fluid were prepared under anaerobic conditions³². The aerobic count was made by plating 10 µl of the appropriate dilution on nutrient agar in air and incubating at 39 °C for 48 h. The total anaerobic count was performed by the method described by Hobson³³ using anaerobic roll tubes³⁴. Ammonia was determined by the method of Weatherburn³⁵. Urease was measured by ammonia production from hydrolysis of urea¹¹ by rumen fluid and by excised epithelial papillae at 39 °C. Bacteria isolated from the highest dilutions (10⁵ and 10⁶) were assayed for ureolytic activity by method (a) of Cook¹¹. The units of activity are µmol NH₃ released per min per ml rumen fluid or per g wet papillae. Figures in parentheses are the number of determinations from which the mean values were calculated.

100 mg wet weight tissue in 2 ml anaerobic medium). The numbers of bacteria attached to the wall of sheep fed by infusion ranged from 0.3×10^7 to 4.3×10^8 per g wet weight tissue, similar to the 4.4×10^7 to 2.2×10^8 per g wet weight tissue found on the wall of a hay-fed sheep. Of bacteria isolated anaerobically from the wall of sheep fed by infusion, 73% also grew aerobically, and 5/37 isolates were ureolytic, indicating that the fluid and wall populations were of similar composition, and that the wall population of infused sheep was fairly similar to a normal animal^{1,5}. Urease activity associated with the wall of infused sheep was also similar to that of normal sheep (Table 1).

The ureolytic bacteria isolated from the fluid and wall were all Gram-positive, catalase-positive, facultative cocci, and so were probably staphylococci or micrococci, the most frequently isolated ureolytic rumen bacteria^{11,16-21}. Like other isolates^{11,21}, urease activity of many of our cultures was lost on repeated subculture. It is not surprising that ureolytic staphylococci/micrococci were isolated, since several species in these genera are ureolytic²², and since a selective medium for growth of staphylococci²³ gave counts which ranged from 0.4% to 27.2% of the total aerobic count for rumen fluid and 11.7% to 14.1% of facultative epithelium-bound bacteria in our infused

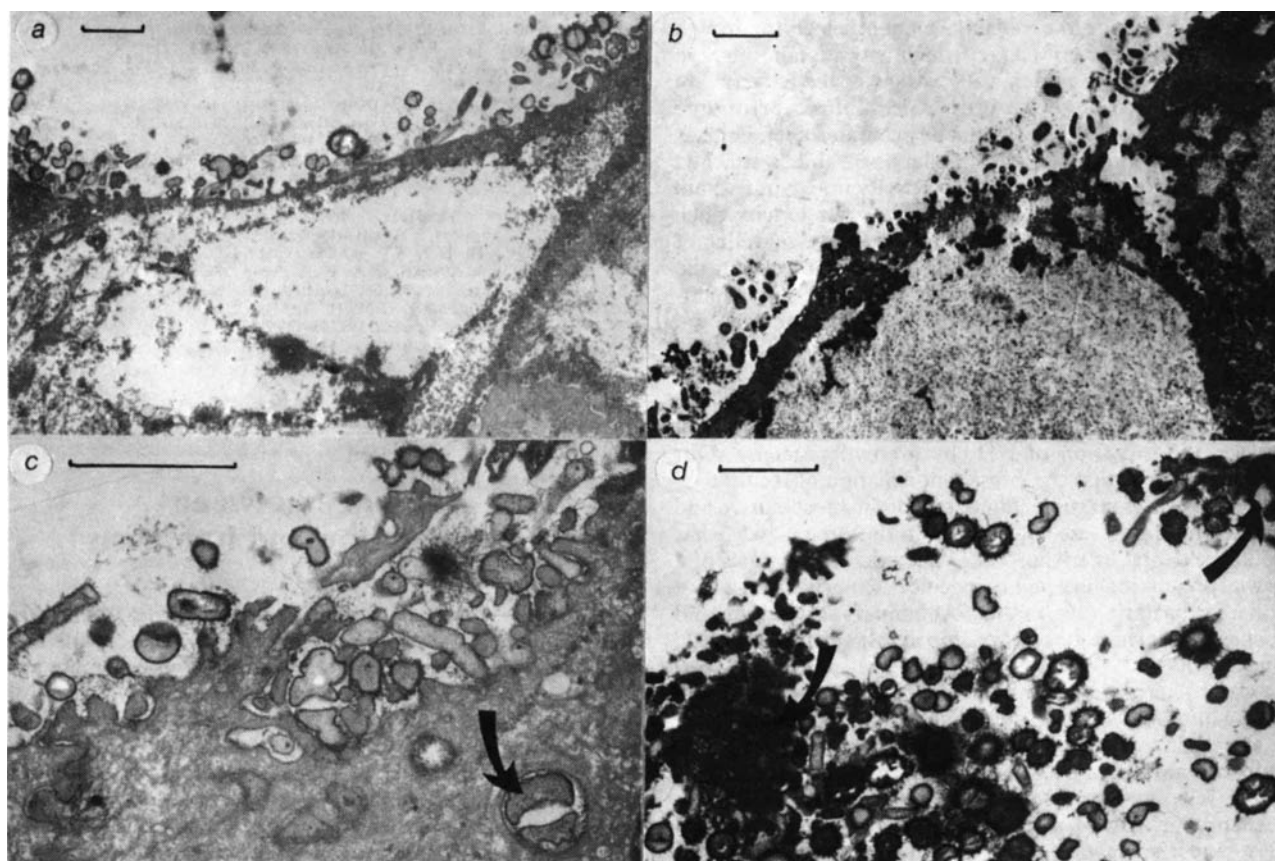


Fig. 1 Microbial colonisation of rumen epithelial tissue in sheep fed normally and by abomasal and ruminal infusion. Individual rumen papillae were bisected longitudinally and fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 followed by 1% osmium tetroxide in 0.028 M acetate veronal buffer pH 7.4. Papillae and samples of rumen fluid from infused sheep were also fixed in solutions containing ruthenium red³⁶. All samples were dehydrated through a graded series of ethanol solutions and embedded in Araldite or Spurr's resin; sections were stained with uranyl formate and lead citrate. Epithelial cells from the rumen wall of infused sheep showed normal surface colonisation (a), similar to that found in control sheep fed on hay (b), despite the absence of feed particles in rumen contents. Bacteria colonising the rumen epithelium of infused sheep showed widespread excavations of the surface layer (c) together with isolated examples of much deeper penetration (arrow). Sloughed epithelial cells (arrows) in the rumen liquor of infused sheep (d) supported a bacterial population similar to that found on the surface of rumen papillae. Scale bars, 2 µm.

sheep. Two similar determinations for the rumen wall of a hay-fed sheep gave proportions of 5.2 and 13.3% of the total aerobic count to be staphylococci.

The white material suspended in infused rumen liquor was identified microscopically to be sloughed epithelial cells. Electron microscopy revealed a heavy bacterial colonisation of the cell surfaces, as found in biopsy samples of epithelium from infused sheep (Fig. 1a) and in normal animals⁴ (Fig. 1b). Considerable bacterial invasion of the dead tissue was also evident in biopsy samples of epithelium (Fig. 1c) and rumen fluid contents (Fig. 1d) from infused sheep. Invasion of epithelial tissue also occurs in normally fed ruminants⁴.

The experiments described here show that the bacterial population of the rumen wall can provide a normal ureolytic activity in the absence of the normal flora of rumen fluid. To determine whether the normal flora can carry out ureolysis at a normal rate in the absence of the facultative wall population, gnotobiotic lambs were inoculated with a range of typical rumen bacteria, as described by Mann and Stewart²⁴ except that no facultative bacteria were included. Urease activity in the rumen fluid was zero, and urea was present at a concentration of 5 mM. The NH₃ concentration was <1 mM. While this finding does not necessarily mean that rumen anaerobes are not ureolytic—the strains necessary may not have been included in the inoculum, although a wide range of organisms was used—it indicates that the facultative population may be largely responsible for ureolysis in normal circumstances.

We now envisage the bacteria of the rumen wall to be a separate and potentially independent population which may survive on nutrients passing through the rumen wall and by digestion of dead epithelial tissue. By its very nature, an aerobic-anaerobic interface, the wall environment selects for growth of oxygen-tolerant bacteria, a fact which may explain the high oxidative activity of the rumen wall²⁵. Many of the bacteria are also adapted to hydrolyse the urea which diffuses across the rumen wall. These bacteria additionally occur in rumen fluid, as a result of the shedding of dead epithelial tissue and its attached bacteria. This process confers urease activity on a rumen fluid population which otherwise would have little or no activity. Such a property is of considerable importance, because recycling of endogenous nitrogen as urea entering the rumen through the wall and in saliva, and the economically important supplementation of low-protein ruminant feeds by urea, both rely on ureolysis by rumen bacteria^{26,27}. Ureolysis in rumen fluid results in the release of NH₃, the major source of nitrogen for rumen bacteria involved in the degradation of plant cell walls,²⁸ so clearly the utilisation of plant material in a low-protein feed will depend on the provision of NH₃ by ureolytic bacteria. Our results demonstrate that the consistent isolation of facultative, ureolytic bacteria from rumen fluid, one of the most elusive and least understood of classes of bacteria in the rumen^{11,21,29}, has not been accidental or trivial. These bacteria are products of a stable wall population, in a highly specific ecological niche where the adherent bacteria rely on the host animal for nutrition and the host uses the symbiotic relationship in respect of an essential enzyme activity.

The wall bacteria may also influence the rumen ecosystem by regulating urea flux across the rumen epithelium. Work on sheep and cattle suggests that entry of urea into the rumen across the rumen wall increases when the ruminal ammonia concentration falls⁷⁻⁹. From the apparent inverse relationship between urease activity and ammonia concentration seen in Table 1, and also in the results of Chalupa *et al.*¹⁴, we can speculate that this feedback control is mediated by the effect of ammonia on urease activity. At high rumen NH₃ levels, removal of urea by urease from the inner face of the rumen wall will be slower than at low NH₃, and consequently the rate of diffusion across the wall will be reduced^{7,30,31}.

Thus, the population of bacteria attached to the rumen wall may not only be a vital component in the recycling of endogenous nitrogen, it may also control the rate at which it occurs. In this way, a numerically small, independent population of

bacteria bound to the rumen wall can have a profound effect on the interaction between the host animal and a second, much larger population of symbiotic bacteria in rumen fluid.

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A major difference between transdetermination and homeosis

IN *Drosophila*, the adult structures arise from nests of larval cells called imaginal disks, whose state of determination with respect to disk type is usually fixed. However, two processes are known which can change determination from one disk type to another; one occurs *in situ* and is due to homeotic mutations, and the other is seen after culture of disk material and is called transdetermination. Similarities between the two, notably the lack of intermediate states, have led to the idea that they are different aspects of the same phenomenon^{1,2}. The existence of homeotic mutations argues that determination in normal development is underlain by a genetic switching mechanism^{3,4}; transdetermination could result from epigenetic changes in this system. If this is so then the two phenomena should have characteristics in common. Studies of homeotic mutants have shown that a structure in one disk is always transformed into a specific structure from the other disk^{5,6}; for example clones of *bithorax* in an anterior dorsal position in the haltere show anterior dorsal wing structures⁵. This is used as a criterion of homology, and also indicates that all disks use the same positional information⁷.

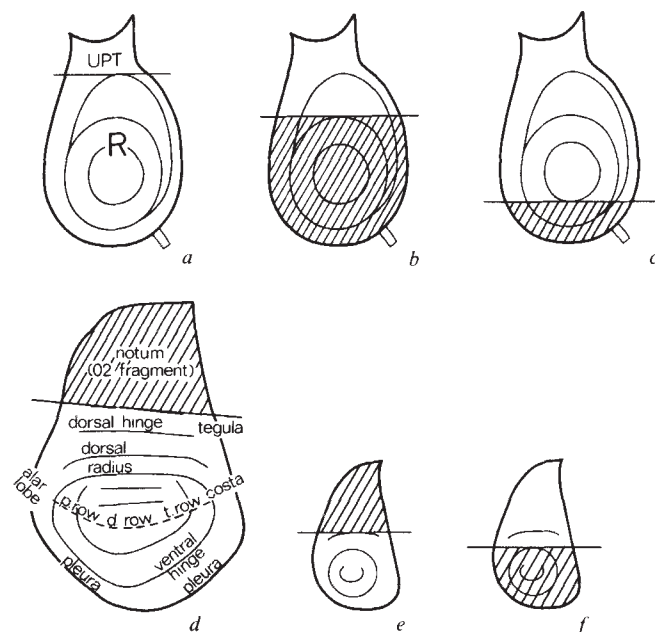


Fig. 1 *a*, The UPT (upper prothorax) fragment of the foreleg disk, which has a high level of transdetermination; the rest of the disk (R) has almost none (Table 1). *b*, This fragment (hatched) is rather smaller than the R fragment and shows no transdetermination. *c*, This fragment stimulates regeneration in the O2 wing fragment. *d*, Fate map of the wing disk, adapted from ref. 14 and showing the O2 fragment. P. row, posterior row of hairs; d. row and t. row, double and triple rows of bristles. *e*, Metanotum fragment of the haltere disk. This fragment does not induce regeneration in the O2 wing fragment. *f*, This fragment induces regeneration in the O2 wing fragment.

Differences between disks must be due to different interpretation of this information, mediated in some way by the wild-type alleles of the homeotic genes. It is not yet known whether transdetermination also shows position-specific transformation; if the two phenomena are closely related then this should be the case. It is often assumed to be so, because there have been reports of transdetermination between leg and antenna in which structures such as claw and arista, thought from homeotic mutants to be homologous, are found adjacent to

one another in implants¹. However, these findings could also be explained by non-homologous transdetermination followed by either movement together of homologous structures due to preferential cell affinities, or intercalary regeneration between the non-homologous structures. Also, regeneration before transdetermination can give misleading results. One cannot simply ask to what structures a given disk fragment will transdetermine as the fragment might regenerate the rest of the disk first, and non-regenerating (that is duplicating) fragments do not appear to transdetermine (unpublished observations, and ref. 8). One solution to these problems is to find a disk in which transdetermination occurs only in one part, and this is the case in the foreleg disk. The experiments reported here were designed to find whether this part transdetermines to a homologous part of the wing disk.

The cells in the foreleg disk capable of transdetermination are confined to the upper half⁹ and perhaps to the presumptive upper prothorax only (UPT, Fig. 1*a*). *Ebony*¹⁰ UPT fragments were dissociated with reciprocal fragments (R) of *yellow*; *multiple wing hairs*¹⁰ genotype. The cells were reaggregated, cultured in adult females to allow growth, and induced to metamorphose. The R fragment was found to have a very low level of transdetermination while the UPT had a high level (Table 1). The *y;mwh* UPT fragments and *e* R fragments were also dissociated together and gave similar results. In a second experiment a fragment smaller than the R fragment (Fig. 1*b*) showed no transdetermination at all (0/20). S. Strub (personal communication) has obtained similar results independently.

Table 2 shows the wing structures differentiated by the UPT. Wing blade, pleura and wing margin structures (double and triple rows) occurred at high frequency. Notum did not appear and nor did other nearby structures such as dorsal hinge, tegula or alar lobe. This same pattern is observed in non-dissociated forelegs (unpublished observations, and ref. 11) so the absence of these structures is not simply due to their disappearance after the proximodistal growth which occurs in dissociations¹².

Table 2 Wing structures differentiated by dissociated foreleg disks

Wing blade	75	Costa	0
Posterior row	8	Tegula	0
Double row	30	Alar lobe	0
Triple row	18	Dorsal hinge	0
Pleura	52	Notum	0
Ventral hinge	9		

The figures refer to percentage of metamorphosed implants containing wing tissue. See Fig. 1*d*, for location of presumptive structures in the wing disk.

Table 1 Transdetermination levels in UPT and R fragments compared with entire foreleg

Dissociated tissue	No. of metamorphosed implants	Average no. of wing vesicles per implant	Estimated percentage transdetermination in each fragment by area
Entire foreleg	36	3.0	20
<i>y;mwh</i> UPT	41	1.1	50
<i>e</i> R		0.2	3
<i>e</i> UPT	37	0.9	50
<i>y;mwh</i> R		0.1	2

The *y;mwh* UPT and *e* R fragments were dissociated together, as were the *e* UPT and *y;mwh* fragments. Dissociation was used for two reasons. First, it stimulates growth, which is a known requirement for transdetermination; second, if the R fragment were not dissociated, it might regenerate the UPT and then transdetermine; dissociation prevents proximal tissue from being regenerated⁹. UPT, upper prothorax (Fig. 1*a*); R, remainder (leg minus UPT). The tissue was dissociated with a microstirrer for 2 min in Ca-free *Drosophila* Ringer with 0.01% collagenase; the reaggregated cells were cultured in adult females for 7 d and transferred to mature larvae for metamorphosis. Male forelegs were used in these experiments; female forelegs behave similarly. The area of transdetermined tissue was estimated by eye to about 10%.

Because no homeotic transformation between wing and leg is known, stimulation of regeneration was used to establish homologies between the two disks; intercalary regeneration will occur between fragments of different disks in the same way as it does between different fragments of the same disk¹³. Regeneration of wing blade from the O2 fragment¹⁴ of the wing disk (presumptive notum, Fig. 1*d*) was used as an assay. When mixed with this fragment the UPT was found to be unable to stimulate it to regenerate wing blade (1/27), while a fragment from the opposite end of the leg disk (Fig. 1*c*, hatched) did so in 8/12 cases. Thus by the criterion of intercalary regeneration, the UPT is homologous to the notum. However, as noted above, the UPT does not transdetermine to notum.

Homology experiments using intercalary regeneration have not been carried out before and the validity of the technique was checked using mixtures of wing and haltere, whose homologies are known from mutations at the *bithorax* locus⁵. These gave the expected results; 1/15 from the O2 with a homologous haltere region (Fig. 1*e*) and 12/15 from the O2 with a haltere fragment from the opposite end (Fig. 1*f*).

It seems that, at least in the case of the foreleg disk, transdetermination is not position-specific. There are suggestions

that the same may be true of the second leg. This leg transdetermines to the same wing structures as the foreleg does, and in particular to ventral hinge. I have found ventral hinge in several implants of second leg lying adjacent to the sternopleural bristles, which are on the upper mesothorax and therefore not homologous to the ventral hinge region of the wing. It is possible therefore that the observation may point to a general principle.

These results indicate that the relationship between transdetermination and homeosis may be less close than is generally thought. Note that no homeotic mutation causing a transformation between dorsal and ventral disks is known¹⁵. The foreleg to wing transdetermination does this, and so do several others, for example antenna to wing¹⁶, wing to leg¹⁷, and second leg to wing (unpublished observations). These could act by changing the activity of as yet undiscovered genes. But in the case of the foreleg to wing transdetermination, this would mean affecting both this dorso-ventral gene and an intersegmental gene, and also changing positional value. As this is the most frequent of all transdeterminations this does not seem very plausible. Alternatively, transdetermination could act by a completely different mechanism which does not involve homeotic genes.

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Epistatic gene interactions in the control of division in fission yeast

THERE is currently much interest in the mechanism which controls the timing of cell division. Certain features of the control have been found to be common to a variety of eukaryotes. In particular, the importance of cell size as a parameter affecting cell cycle progress has been reported for mammalian cells^{1,2} and for several single-celled eukaryotes³⁻⁶. Another feature common to several systems is that growth conditions have a direct effect on the timing of division cycle events⁷⁻⁹, and on cell size^{9,10}. In the fission yeast *Schizosaccharomyces pombe*, both cell size⁶ and nutritional conditions⁹ have been shown to affect cycle kinetics. The organism has been used extensively as a model eukaryotic system, largely because of the ease of measuring cell size and because division occurs by binary fission¹¹. More recently, its genetic tractability has led to the isolation of cell division cycle (*cdc*) mutants¹², and also of *wee* mutants altered in the control coordinating growth with the division cycle¹³⁻¹⁵. The existence of such control mutants allows a more direct approach

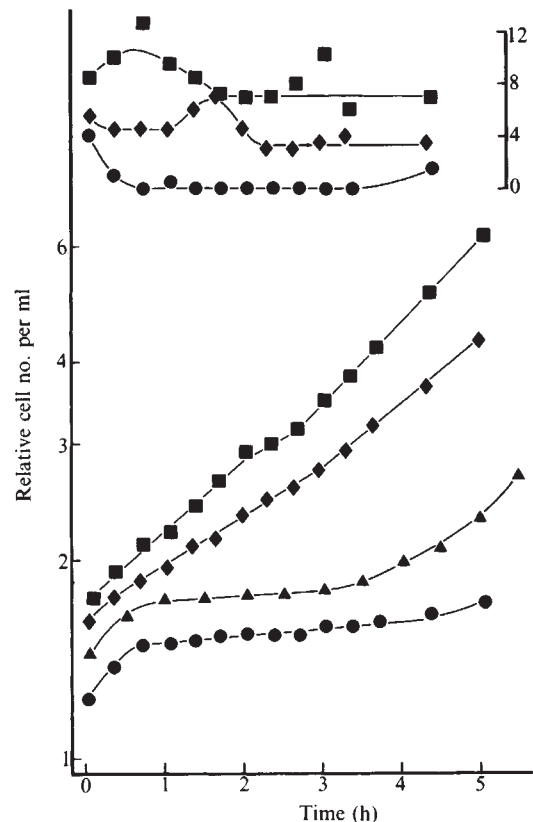


Fig. 1 Effect of temperature shift on division in *S. pombe* strains. Asynchronous cultures of the strains were grown at 25 °C in modified Edinburgh minimal medium, EMM2 (ref. 12) and were shifted to 35 °C at time zero. Samples were taken to estimate cell number per ml of culture (bottom) and the percentage of binucleate cells (top). Cell no. per ml is plotted semilogarithmically on an arbitrary relative scale, such that actual cell no. per ml and the scale values are related by factors of: *cdc25.22* (●), 10^6 ; *wee1.6* (■), 1.86×10^6 ; *wee1.6cdc25.22* (◆), 1.5×10^6 ; *wee2.1cdc25.22* (▲), 2×10^6 . Methods have been described previously¹².

to the investigation of the molecular basis of division control, in contrast to the indirect methods used in other systems^{4,16-18}. *wee* mutants are so far unique to *S. pombe*. The most conspicuous property of *wee* mutants is their reduced cell size^{13,14}. Analysis of these mutants^{15,19} and other evidence⁹ has shown that control over cell division timing normally acts at entry to mitosis. As the function of a number of *cdc* genes is specifically required for mitosis¹², interactions between *wee* and *cdc* mutants which affect mitosis might be expected. I report here that the mitotic defect caused by a defective *cdc25* allele is suppressed in *wee* mutants. Suppression by *wee1* mutants is almost complete, while the *wee2.1* mutation is a less effective suppressor. The significance of these findings for genetic models of the control of mitosis is considered.

The gene *cdc25* controls an event either in or before mitosis. Strains having either of the mutant alleles *cdc25.22* or *cdc25.M51* are able to undergo mitosis and cell division at 25 °C. However, when shifted to 35 °C, cells accumulate in G2 with a single nucleus. Both mutations are recessive to the wild-type allele in that the heterozygous diploids *cdc25/cdc25+* are able to divide at 35 °C. Both *cdc25* mutations were originally isolated from strain 972 (*cdc+*, *h-*) after nitrosoguanidine mutagenesis. *cdc25.22* was obtained (P. Thuriaux, M. Sipiczki and P.F., unpublished) from a previously described sterile strain ED22 (ref. 12); *cdc25.M51* was isolated by Dr K. Nasmyth (unpublished). Both strains used are derivatives of the originals, obtained by backcrossing to strain 975 (*cdc+*, *h+*).

Mitosis was rapidly inhibited when an asynchronously growing *cdc25.22* culture was shifted from 25 to 35 °C (Fig. 1). Those cells which were close to or had completed mitosis at the time of

shift completed one residual cell division, after which cell division ceased (Fig. 1). The fraction undergoing residual division was 25%, corresponding to a transition point in the cell cycle of 0.68, shortly before mitosis at 0.75 (ref. 12). The protein content of the culture continued to increase, now with a doubling time of $2\frac{1}{2}$ h, and the cells elongated (data not shown). Shifting a wild-type culture in the same way gives a similar pattern of protein increase but in this case cell number continues to increase after a short plateau¹³. The same shift-up experiment was carried out with *wee1.6cdc25.22* and with the parent *wee1.6* (Fig. 1). In the double mutant, cell division continued in a similar manner as in the *wee1.6* culture. In neither strain did the binucleate index fall to zero after shift to 35 °C and, despite some fluctuation, remained on average near its original value (Fig. 1). In both cultures the protein content increased with cell number, with doubling times of around 3 h. These results show that the rate of entry into mitosis in *wee1.6* cells is scarcely affected by the presence of the mutant *cdc25* allele; that is, the *cdc25* defect is suppressed by the presence of the *wee1.6* lesion. Indeed, the double mutant is able to form colonies on solid medium at 35 °C, and grows in liquid culture with a similar doubling time to *wee1.6* at 25 or 35 °C (Table 1). This shows that the suppression of the *cdc25* defect lasts for many generations and is not a short term effect only observed soon after shift to 35 °C. Furthermore, the cell size of the double mutant grown in steady state at 35 °C is only slightly greater than that of *wee1.6* (Table 1). Therefore, *wee1.6* almost completely suppresses the effects of *cdc25.22*, eliminating its *cdc* phenotype at 35 °C, and reducing cell size in the double mutant to close to that of *wee1.6* itself.

Table 1 Cell length at division, mean protein content per cell and doubling time of *S. pombe* strains

Strain	Growth at 25 °C			Growth at 35 °C		
	DL	PC	DT	DL	PC	DT
<i>cdc25.22</i>	20.2	21.0	210	—	—	—
<i>cdc25.M51</i>	26.4	29.6	240	—	—	—
<i>wee1.6</i>	8.2	7.5	305	8.1	8.5	170
<i>wee1.6cdc25.22</i>	8.4	7.8	300	9.2	10.6	180
<i>wee1.50</i>	10.9	9.7	240	7.9	6.9	160
<i>wee1.50cdc25.22</i>	15.9	12.9	250	9.5	9.9	165
<i>wee2.1</i>	9.2	8.4	250	7.7	6.6	155
<i>wee2.1cdc25.22</i>	10.6	8.6	280	24.4	28.8	210
972 (wild type)	12.8	11.5	240	13.9	12.6	140

DL, cell length at division (μm); PC, mean protein content per cell in asynchronous culture (pg); DT, exponential doubling time (min). See ref. 12 for methods.

To determine how widespread the suppression of *cdc* phenotype by *wee* mutants is, a series of double mutants of constitution *wee1.6cdc*[−] were constructed and tested for their ability to undergo cell division at 35 °C on agar medium. Only in the case of the other known *cdc25* allele, *cdc25.M51*, was the cell cycle block suppressed: the mitotic defects¹² caused by mutant alleles of *cdc1* (three alleles tested), *cdc2* (10 alleles) and *cdc13.117* were unaffected. The mutations *cdc10.129*, defective in the initiation of DNA replication²⁰, and *cdc3.6*, defective in cell plate formation¹², were unaffected by the presence of *wee1.6*. Therefore, of those mutations tested, *wee1.6* suppresses only *cdc25* lesions. The gene specificity argues against the suppression being caused by mis-sense suppression at the translational level. Other *wee1* mutations show the same suppression of *cdc25* phenotype: those tested include *wee1.1* and *wee1.3*, which are phenotypically similar to *wee1.6* (ref. 1), and *wee1.50* (Table 1), which is a temperature-sensitive allele¹³. To check the genotypes of double mutants, several were backcrossed to the wild type of opposite mating type, and the segregants analysed by tetrad analysis. All the results were consistent with the presence of *wee1* alone being sufficient to suppress the *cdc25* phenotype. Thus the suppression of *cdc* phenotype is gene- though not allele-specific.

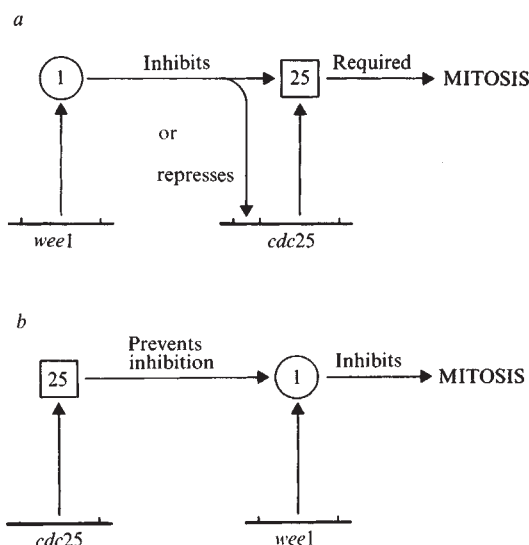


Fig. 2 Models to explain the interaction between *wee* genes and *cdc25*. See text for detailed explanation: for simplicity *wee2* is omitted. *a*, Functional *cdc25* product is required for mitosis. Either the synthesis or activity of this product is inhibited by functional *wee1* product. *b*, Functional *wee1* product inhibits mitosis. The inhibition is prevented by functional *cdc25* product. ① Is the *wee1* gene product, and ② the *cdc25* gene product.

The effect of the *wee2.1* mutation on *cdc25.22* is different from that of *wee1* lesions. When *wee2.1cdc25.22* was shifted from 25 to 35 °C, the behaviour of the cells was initially similar to that of the *cdc25* parent. About 25% of the cells underwent a residual division, which was followed by a plateau in cell number for about $2\frac{1}{2}$ h (Fig. 1). After this, however, cells started to divide again, and eventually a rather slow steady-state growth of the strain occurred at 35 °C (Table 1). In this case the cells were about twice the size of wild-type cells (Table 1), so *wee2.1* does not completely suppress the *cdc25* lesion.

In what way are the interactions between *wee* and *cdc25* mutations informative about the control of mitosis and of cell size? The genes *wee1*⁺ and *wee2*⁺ code for negative and positive elements respectively in the control over mitosis¹⁵. On the other hand, *cdc25*⁺ is a gene whose product is apparently required for mitosis. This is probable as both mutant *cdc25* alleles are recessive to the wild type and hence probably code for products with reduced or absent function.

Three factors relevant to the interactions must be considered: cell size itself, the function of the *wee*⁺ gene products, and the activity of the *cdc25* gene product. *wee* mutations reduce cell size, while *cdc25* mutations increase it substantially at 25 °C. At 35 °C, *cdc25* mutants are unable to divide: they can therefore be considered to divide at infinite size. In *wee*[−]*cdc25* double mutants, division size is reduced compared with the *cdc* parent in all conditions. *wee1* mutations have a stronger effect than *wee2.1* in reducing the cell size of *cdc25.22*.

The interactions between *cdc25* and *wee* mutations can be considered in two distinct ways, depending on the direction of information flow between the two systems (Fig. 2).

(1) The *wee1/wee2* system could regulate the activity of the *cdc25* product, at the level of either synthesis or action. Lesions within the *wee* system would bring about an increase in *cdc25* activity. Provided that the increase were large enough, and that the mutant *cdc25* products had some small activity at 35 °C, this could be sufficient to allow mitosis (Fig. 2). In contrast, the completeness of suppression by *wee1.6* suggests that the increase in *cdc25* activity might have to be manyfold. Furthermore, it is perhaps improbable that two different *cdc25* mutations would each code for a product with significant residual activity at 35 °C, particularly as neither mutant is very 'leaky' at this temperature. (The *cdc25* mutations are thought to code for

different products as they are of independent origin, and have different phenotypic effects on cell size at 25 °C (Table 1.)

(2) A more plausible suggestion is that the *cdc25*⁺ product is only required for mitosis when the *wee* system is intact. In this model (Fig. 2), the *wee1*⁺ product inhibits mitosis, perhaps through the *wee2*⁺ gene¹⁵. This inhibition is normally prevented by the wild-type *cdc25*⁺ product. In *wee1* mutants, mitosis is 'de-inhibited' and takes place at a reduced cell size. In the absence of a functional *wee1*⁺ product, the activity of the *cdc25* product can have no effect on entry into mitosis. *wee1* mutations are therefore epistatic over *cdc25* and the cells are small, irrespective of the *cdc25* allele. In *cdc25wee*⁺ cells the wild-type *wee1*⁺ product inhibits mitosis more effectively than usual, as the mutant *cdc25* product is less effective at preventing the inhibition. Large, or ultimately, non-dividing cells will result. Similar arguments can be used to explain the *wee2/cdc25* interaction. The suggestion that *cdc25*⁺ affects mitosis through the *wee* system follows classical interpretations of epistatic relationships between genes. There is also a parallel situation involving the *wee1* gene and the effect of nutrient conditions on the mitotic control in *S. pombe*. The nutritional modulation of cell size at mitosis which occurs in the wild type⁹ is abolished in *wee1* mutants¹⁹. I suggest that *wee1*⁺ may act as a central control point in the cell, monitoring several factors which affect entry into mitosis, including *cdc25* activity, nutritional status and cell size.

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Isolation of a spermatozoid-releasing and -attracting substance from female gametophytes of *Laminaria digitata*

SEVERAL dioecious marine brown algae have been valuable in studying chemical communication during sexual reproduction. For example, female gametes of *Ectocarpus siliculosus*, *Cutleria multifida* and *Fucus serratus* secrete olefinic hydrocarbons into the seawater to attract male gametes^{1–3}. A more complex system is found in the order Laminariales, which includes the large kelps. Mature female gametophytes in members of this group

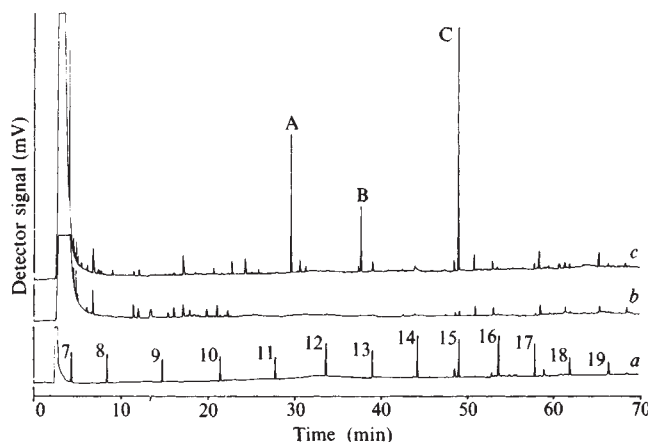


Fig. 1 Capillary gas-chromatographic separation of extracts from female gametophytes of *Laminaria digitata*. *a*, normal alkanes ranging from heptane to nonadecane separated in identical conditions; *b*, 3-d-old suspension without eggs, extracted for 22.5 h; *c*, suspension containing 8.7×10^6 eggs, extracted for 24 h. *b* and *c*, same attenuation. Chromatography conditions: splitless injection technique, splitless period 30 s. Glass capillary column 45 m $\times$ 0.16 mm, coated with OV 101. Carrier gas: 0.6 ml min⁻¹ H₂. Injector and FID at 200 °C. Temperature programme: 5 min at 5 °C; 2.5 °C min⁻¹; 5 min final temperature at 150 °C.

secrete highly volatile material with spermatozoid-attracting activity, which also induces explosive discharge of antheridia⁴. We report here a study of this volatile material. It is now possible to attribute spermatozoid-releasing and -attracting activity to one specific compound.

Clonal cultures of one male and one female gametophyte of *Laminaria digitata* (Huds.) Lamour, isolated from Helgoland were used. Mass cultures of female gametophytes were maintained in red light in conditions described previously⁴. Batches containing 5–10 g fresh weight filamentous female gametophytes were ground in a mortar and suspended in an extraction flask with 1.8 l of culture medium. This suspension was agitated with a magnetic stirrer and exposed to continuous white light (2,500–3,500 lx, Osram fluorescent lamps L 40 W/19) at 12 °C. On several successive days the algal fragments were allowed to sediment and the supernatant was replaced by fresh medium. Cultures maintained in this way developed oogonia, and free eggs appeared in the suspension from the eighth day.

Volatile compounds were extracted by the closed-loop stripping method⁵ and adsorbed on a filter consisting of 2 mg of activated carbon. Suspensions containing 10^6 – 10^7 eggs were subjected to the extraction procedure for 24 h, then left for one to several days in optimum light conditions. When a high count of eggs had built up again, another extraction was started. Adsorbed compounds were desorbed from the filter with 30 μ l dichloromethane, and the resulting solution was glass capillary gas chromatographed on a Carlo Erba Fractovap 2900 with two columns containing stationary phases with different polarity (OV 101, 45 m; UCON 75-H 90000, 43 m) and with two flame ionisation detectors.

The detector signals were recorded and integrated with a Hewlett Packard Integrator 3385 A. For assay, various fractions were recondensed in a U-shaped trap containing SiO₂ particles (Spherosil, type XOB-075, Serva) and cooled with dry ice. Spermatozoid-releasing and -attracting activity adsorbs on these particles and can be assayed by testing their effect on mature male gametophytes⁴.

Extraction of suspensions containing immature female gametophytes without eggs yielded trace amounts of several compounds (Fig. 1*b*), most of which were also found in blank runs with the culture medium alone. Suspensions containing eggs and empty oogonia yielded three major compounds clearly produced by those structures (Fig. 1*c*). These three compounds were separated on the UCON column and the respective fractions (A, B and C) were recondensed in traps containing

Spherosil. Addition of particles containing fraction B to mature male gametophytes induced mass spermatozoid release, followed by large haloes of spermatozoids around the sources (Fig. 2). No noticeable release or attraction of spermatozoids was seen with particles containing fractions A or C, or with mixtures of both. Clearly, both spermatozoid release and attraction are caused by fraction B. Gas-chromatographic comparison of retention times and co-injection with authentic samples on both columns enable us to identify fractions A and C. A is the cyclic olefin ectocarpin, which acts as the male attractant in the brown alga *Ectocarpus siliculosus*¹. C is *n*-pentadecane. The identity of B is unknown. It has Kovats indices of $1,265.9 \pm 0.3$ ($50 \pm 0.5^\circ\text{C}$) on OV 101 and $1,621.2 \pm 0.5$ ($80 \pm 0.5^\circ\text{C}$) on UCON 75-H 90000. In many extracts from suspensions containing eggs, *n*-pentadecane was always the most prominent fraction, whereas ectocarpin and compound B were present in smaller concentrations. Nine different runs were used to obtain quantitative estimates of substances produced by 10^6 eggs within 1 h. The following average values were obtained: ectocarpin 7.0×10^{-9} g; fraction B 4.8×10^{-9} g; *n*-pentadecane

1.6×10^{-8} g. We are now trying to accumulate sufficient compound B to identify it.

The large difference in Kovats indices of compound B indicates a highly polar molecule. So far, all signal molecules known in brown algae have acted as attractants only. *Laminaria* is the first case where one substance has two different functions—release and attraction of male gametes. The extraction procedure cannot reveal the source of the substances because mature oogonia, empty oogonia and free eggs are all present in the suspension. Nevertheless, it seems likely that compound B is secreted by the eggs themselves because the spermatozoids must be directed to the eggs. Ectocarpin and *n*-pentadecane may be associated with other structures such as the portion of residual cytoplasm which is frequently left behind after the egg has left the oogonium⁶. It cannot be ruled out, however, that *n*-pentadecane is a normal constituent of eggs and is set free during their deterioration. It is known that *n*-pentadecane is the most prominent paraffin in whole thalli of *Laminaria digitata*⁷.

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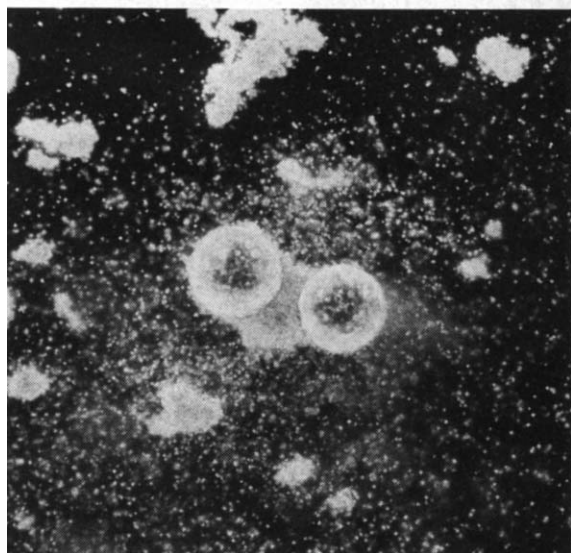
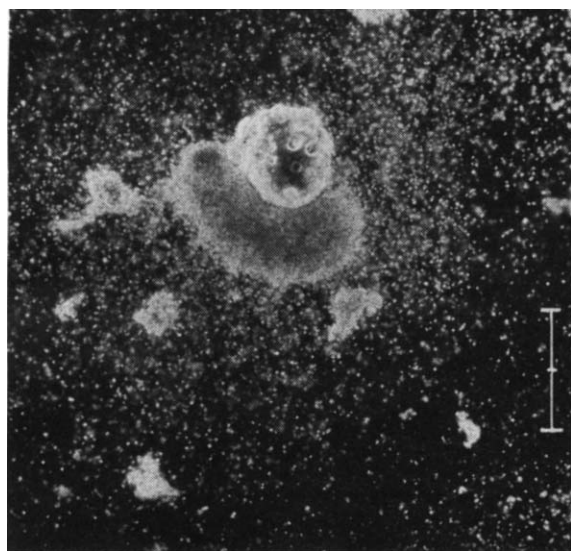


Fig. 2 Effect of spheric Spherosil particles upon male gametophytes (diffuse bodies of various sizes and shapes) of *Laminaria digitata*. Mass release of spermatozoids and their subsequent accumulation around the Spherosil spheres. Occasionally, especially large haloes of intensely active clouds of spermatozoids are formed around certain parts of the spheres (top), or in the area between two particles (bottom). Each particle contains 6×10^{-14} g of compound B. Dark field, flashlight exposure. Scale bar, 200 μm .

Differential susceptibility of the axilla and groin of the mouse to chemical oncogenesis

INCIDENTAL to investigations of chemical oncogenesis, we have observed that 3-methylcholanthrene (3-MC)-induced subcutaneous sarcogenesis occurred more rapidly in the axillary than in the inguinal region of the mouse. Recently Auerbach *et al.* have described what may be a similar phenomenon—a marked tendency for tumour transplants to grow better cephalically^{1,2}.

The basic method in our studies was to implant carcinogen-containing wafers subcutaneously. The wafers were made from Millipore filter material, usually, but not necessarily, of 0.45- μm porosity. Strips of filter were dipped in solutions of molten embedding paraffin (Paraplast) which contained various concentrations of 3-MC. Temperature was carefully controlled by an oil bath to prevent overheating and consequent decomposition of the 3-MC. After cooling, disks of 6-mm diameter were cut with a paper punch and stored in the dark in a refrigerator for up to 1 month. Implantation of the disks was done through small skin incisions while the animals were anaesthetised with a Nembutal alcohol solution. Each animal received four disks, one in each axilla and one in each groin. After implantation of the disks, the animals were observed for tumour production. The resulting tumours are designated 'pre-excision'.

The animals in most experiments were BALB/cByJ females, 4–8 weeks of age (Jackson Laboratory). In experiments done at the Institute for Cancer Research in Philadelphia, the animals were (C57BL/6JNcr $\times$ BALB/cAnNcr)_{F1} females approximately 8 weeks old. In some experiments, the animals were thymectomised 7–10 days before disk implantation and given 450 rad of X rays approximately 24 h before implantation.

Table 1 Numbers of pre-excision tumours arising by axillary or inguinal disks before a tumour had appeared at the other end of the animal

% 3-MC	Genotype	Axillary tumours first/ total no. of tumours	% Axillary tumours first	P values (sign test)	P values (χ^2)
5%	BALB/cByJ φ (X-ray irradiated)	29/36	81%	<0.001	NS†
5%	BALB/cByJ φ	34/38			
"	"	24/27			
"	"	19/20			
	Subtotals	77/85	91%	<0.001	$\begin{array}{c} \uparrow <0.01 \\ \downarrow <0.001 \end{array}$
0.5%	F ₁ *	29/34	85%	<0.001	
	Totals	106/119	89%	<0.001	
0.05%	BALB/cByJ φ	43/51			$\begin{array}{c} \uparrow <0.01 \\ \downarrow <0.001 \end{array}$
"	"	16/22			
"	"	13/23			
	Subtotals	72/96	75%	<0.001	
0.01%	BALB/cByJ φ	12/33	<50%	NS	
	Totals	84/129	65%	<0.001	

* See text.

† NS non-significant.

For unrelated reasons³, when a tumour appeared, the tumour and inciting disk were excised and the animal observed for the appearance of the next tumour at one of the remaining disk sites. In an equal number of as yet non-tumorous mice, the corresponding disk was excised. Tumours arising in mice in which a tumour and disk or a disk had been previously excised are referred to as 'post-excision' tumours.

The data for pre-excision tumours are shown in Table 1. In all experiments in which the concentration of 3-MC in the disks was large, ($\geq 0.5\%$) the tumours usually appeared first by the axillary disks. As the concentration of 3-MC was reduced, the axillary preference, that is the tendency for tumours to appear first by the axillary disks, gradually disappeared; at the 0.01% concentration it vanished.

We will assume here that the observations of Auerbach *et al.* with transplanted tumours are related to the same underlying biological phenomenon as that which we have described. Their studies seem to rule out simple explanations of the axillary preference based on differences in capillary beds, inflammation, or other vascular peculiarities². Their results also suggest that it is a general phenomenon seen with several kinds of tumour transplanted either subcutaneously or intradermally¹, and that the underlying biological gradient slopes in both directions from the anterior thoracic region and from dorsum to ventrum⁴.

Our observation that the axillary preference was critically dependent on the concentration of the 3-MC may be a key to the basis of the phenomenon. It is known that the lower the concentration of the 3-MC, the longer the latent period, the lower the average immunogenicity of the resulting sarcomas when they are transplanted⁵ and the lower the systemic effect on the immune mechanism of the host⁶. The lower immunogenicity of the tumours may be, in part, a function of immunoselection by a relatively intact immune mechanism⁷. But it is clear that even in

the complete absence of immunity, higher concentrations of 3-MC tend to produce tumours of higher immunogenicity⁸. This tendency is still detectable when tumours of equal latencies are compared⁹.

If the critical role of 3-MC concentration were mediated by the differences in the immunogenicities of the resulting tumours, we would expect that measures designed to reduce levels of immunity, other than reduction in 3-MC concentration, would also abolish the axillary preference. However, the results of Auerbach *et al.* argue against the possible role of immunity; they found that the axillary preference, in the case of transplanted tumours, still existed in nude mice and, although their data suggest that it may have been reduced, it was not abolished in animals that had been immunocrippled with 500 rad of γ radiation before tumour implantation¹. Our results, although too small a sample to be statistically significant, also suggest that, although axillary preference in oncogenesis persisted in irradiated-thymectomised animals, it may have been reduced as compared to normal controls, especially among the post-excision tumours with their somewhat longer latencies (Table 2). Furthermore, there is evidence that nude mice possess a rather effective antitumour immunity, albeit without memory and presumably dependent on non-T-cell mechanisms¹⁰. Thus, the persistence of axillary preference in nude mice does not necessarily argue against the immunological hypothesis. We believe, because of the strong effect of the concentration of oncogen (and thus, perhaps, of the immunogenicities of the tumours) and because of the possible partial suppression of the axillary preference in the radiation experiments that the hypothesis that preference is in some way related to immunological phenomena cannot yet be discarded. It could be argued that there may be a cephalo-caudal immunological gradient; however, we are unaware of any evidence for such a gradient. Although it may be

Table 2 Numbers of post-excision tumours arising by axillary or inguinal disks before a post-excision tumour had appeared at the other end of the animal

% 3-MC	Genotype	Axillary tumours first/total no. of tumours	% Axillary tumours first	% Axillary tumours expected first*	P value†
5%	BALB/cByJ φ	73/131	56%	36%	NS
5%	BALB/cByJ φ (X-ray irradiated)	18/41	44%	40%	
			NS		

* % Axillary tumours expected first if there were no axillary preference. This is the proportion of axillary disks that remained at risk for post-excision tumours.

† One tail χ^2 . The test was done after the data were adjusted to compensate for the unequal expectations (36% and 40%) in the two groups.

premature to discard the immunological hypothesis, the fact that lowering the concentration of 3-MC abolished the axillary preference, while immunocrippling had, at most, a very small effect, does imply that most of the effect of 3-MC concentration was probably mediated by some non-immunological gradient, whose effect might have been somewhat magnified by an altered immune response. The nature of this non-immunological gradient is completely unknown, but may bear some relationship to embryonic gradients and to gradients in regenerative capacity¹¹⁻¹⁵.

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Selective effects of cyclosporin A on colony-forming lymphoid and myeloid cells in man

THE properties of the fungus metabolite, cyclosporin A, have suggested its potential as a clinically valuable immunosuppressive agent^{1,2}. Experiments in animals have demonstrated that its suppressive action against cell-mediated and humoral immunity is not accompanied by any appreciable myelotoxicity^{3,4}. In this respect, cyclosporin A contrasts with other immunosuppressants in current use, such as antilymphocyte globulin and azathioprine. There are, however, few data to substantiate a selective toxicity of cyclosporin A against human lymphocytes. Here, we have compared its effects against human lymphoid and myeloid cells, using colony formation by the different cell types as the end point. We found that the compound exhibited far greater toxicity towards a sub-population of T cells than towards either B lymphocytes or haematopoietic precursor cells.

The cyclosporin A (Sandoz) was dissolved in dimethyl sulphoxide (DMSO) and the appropriate dilutions also made in DMSO. Peripheral blood cells were provided by normal volunteers and bone marrow cells by donors for bone marrow transplantation. The mononuclear cells were collected after density gradient separation (Teva)⁵. 'B' cells were obtained from cell lines derived from American Burkitt's lymphoma patients and maintained in this laboratory (manuscript in preparation).

We used these transformed cells as no colony assay for normal human B lymphocytes is yet available.

The cells were plated in semi-solid media in the different culture conditions required for the growth of granulocytic CFU-C (ref. 6), erythroid BFU-E (ref. 7), T-cell⁸ and B-cell colonies. CFU-C were cultured in 0.8% methylcellulose using 10% (v/v) T-cell conditioned medium as a source of colony-stimulating activity⁹ and BFU-E in 0.8% methylcellulose in the presence of 2 units of erythropoietin (Connaught Step III) per plate. Approximately 1% of the B cells formed colonies when they were plated in 0.8% methylcellulose without the addition of any mitogen or stimulating factor. The yield of these colonies was linear over the range $1-20 \times 10^4$ cells per plate. The culture system used for T-cell colony growth gave rise to two morphologically distinct populations of colonies, as described by Rozenszahn *et al.*⁸, one near the surface of the culture and the other near the bottom (top and bottom colonies, respectively).

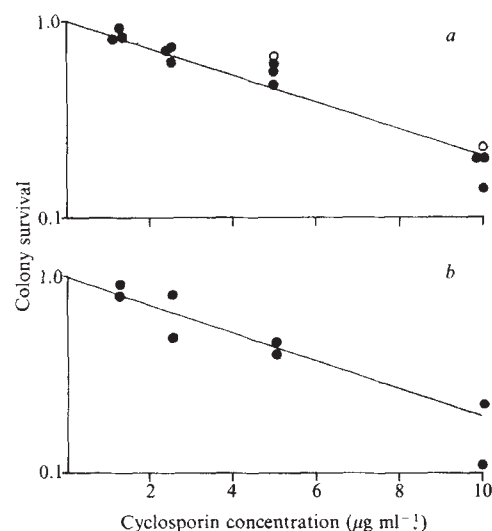


Fig. 1 Effect of cyclosporin A on the growth of: a, CFU-C (●) and BFU-E (○); and b, B-lymphocyte colonies.

The cyclosporin A was added to the plating mixture and all control and experimental plates contained the same amount of DMSO (1 µl per plate). The survival of drug-treated colony-forming cells was expressed as the fraction of the colony yield on the control plates. For these experiments the pooled control yields were 42.0 ± 6.6 CFU-C per plate and 20.0 ± 0.58 BFU-E per plate. One-cm² areas of the plates containing T- and B-cell colonies were counted, giving control yields of 264.7 ± 75.4 top T-cell colonies, 127.6 ± 42.0 bottom T-cell colonies and 63.3 ± 18.9 B-cell colonies.

CFU-C, BFU-E and B-lymphocyte colony-forming cells were not markedly sensitive to cyclosporin A over the dose range used (Fig. 1a, b). However, the two populations of T-lymphocyte colony-forming cells gave surprisingly different results. Figure 2 shows the results from three normal individuals. In two instances, similar reductions in top colony survival were seen with increasing dose and the third showed much greater sensitivity to the compound. The bottom colonies from this individual showed marked insensitivity to cyclosporin A whereas bottom colonies from the other two followed dose-independent plateaus at 10% and 30% survival.

These data confirm the selective effect of cyclosporin A against human T cells and its lack of effect against granulocytic, erythroid and B-lymphoid cells and agree with results obtained from experimental animals^{3,4}.

More intriguing are the different responses of top and bottom colonies to cyclosporin A treatment, and it is noteworthy that, in recipients of allogeneic marrow, graft-versus-host disease is

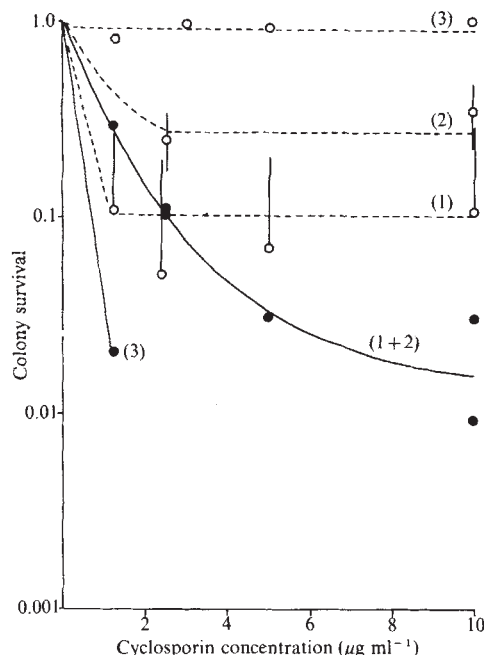


Fig. 2 Effect of cyclosporin A on the growth of T-lymphocyte colonies (top colonies (●); bottom colonies (○); numbers in parentheses refer to the different individuals studied).

accompanied by an increase in top colony-forming cells (unpublished observations). These two types of colonies thus represent subpopulations of T lymphocytes which are separable by their different sensitivities to the compound. The data also suggest that one subpopulation may influence the other, at least *in vitro*, as the greatest sensitivity of top colonies to cyclosporin A was associated with the least sensitive bottom colonies.

No evidence for a difference between top and bottom colony-forming cells has been reported. Although these colonies can be recognised by their gross morphology, the morphology and staining characteristics of the component cells are identical⁸. Rozenszahn *et al.*⁸ found that 50–75% of the cells in the top colonies formed rosettes with sheep red blood cells (SRBC) and, as well as confirming other characteristics, we found that 65% of cells from bottom colonies also formed SRBC rosettes.

We conclude that, as well as offering a non-myelotoxic immunosuppressive activity, cyclosporin A provides a potentially useful tool for the investigation of cultured T-lymphocyte subpopulations.

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Expression of endogenous murine leukaemia viruses in AKR/J starker mice

MICE of the AKR/J strain show high rates of spontaneous thymic lymphoma and are chronically infected with murine leukaemia viruses. Total thymectomy in young mice of this strain results in a marked decrease in the incidence of leukaemia. The finding of athymic mutant mice on the AKR/J background (referred to as starker mice) is of importance in the study of genetic factors which influence virus expression and leukaemogenesis. Here we report that mice of both normal and mutant genotypes vary in the expression of a particular class of murine leukaemia virus. This lower virus expression in conjunction with the absence of a thymus leads to a lower tumour incidence in starker mice.

Congenital thymic aplasia in nude mice has provided a tool to study the role of the thymus in the development of the immune system and in tumorigenesis. Because the original mutation to *nu* occurred in a non-inbred mouse colony¹ much of the early work with nude mice was subject to variability associated with heterogeneity of the genetic background. The nude gene has been backcrossed on to various inbred strains, but the possibility of contaminating genes remains. A new spontaneous autosomal recessive mutation in AKR/J mice characterised by congenital thymic aplasia and lack of hair was found by Eichler². Allelism tests showed the new mutation to be allelic with nude and the mutation was named starker, and given the gene symbol *nu^{str}*. AKR/J-*nu^{str}*/*nu^{str}* mice have few thy-1-positive cells, do not reject allogeneic skin grafts, show a poor humoral response to sheep erythrocytes, and an impaired mitogenic response to T-cell mitogens³. Since thymectomised AKR/J mice do not develop the thymic leukaemia typical of this strain, it could be expected that starker mice would also be refractory to thymic lymphoma development. This hypothesis is supported by our autopsy findings. However, in our colony seven starker mice (5–14 months of age) have developed lymphoreticular neoplasms; five of these tumours were reticulum cell sarcomas (RCS) and the remainder were granulocytic leukaemias. It seems therefore that congenital thymic aplasia in AKR/J mice abolishes the occurrence of typical lymphomas, and permits the development of tumours normally rare or absent in this strain.

The high incidence of spontaneous lymphatic leukaemia in AKR/J mice is associated with the continuous expression of type-C retroviruses throughout life⁴. The endogenous murine leukaemia viruses of AKR/J mice consist of a heterogeneous group of agents. On the basis of host range the MuLVs are divided into (1) those derived from the non-linked dominant loci *Akv-1* and *Akv-2* (ref. 5) and infect both mouse and rat cells (ecotropic virus) and (2) those that can only infect cells of heterologous species (xenotropic virus)⁷. High titres of ecotropic virus are expressed at all ages in these mice, whereas low levels of xenotropic virus are expressed in young mice (<5 months). However, titres of xenotropic viruses were found to increase with age, particularly in the thymuses of preleukaemic mice. A new class of virus with a dual-tropic host range, designated mink cell focus-inducing (MCF) virus was isolated from thymuses of late preleukaemic and leukaemic AKR/J mice⁸. It has been suggested that the MCF virus represents a recombinant between ecotropic and xenotropic MuLVs and that the recombination occurs within the envelope gene which codes for the surface glycoprotein of the virion¹⁰. In addition an N-tropic non-transforming virus that is poorly infectious for mouse cells was isolated from AKR/J leukaemic cells established in culture¹¹. It has been shown that these latter two isolates rapidly accelerate the occurrence of leukaemia in susceptible mice¹². Here we report that starker mice produce both ecotropic and xenotropic viruses, but not recombinant type viruses, suggesting that the

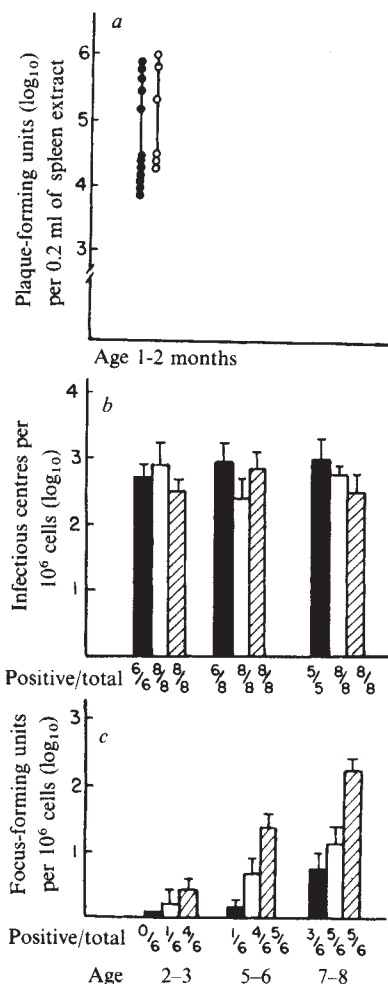


Fig. 1 Expression of endogenous viruses in AKR/J (+/?) and nu^{str}/nu^{str} mice. Infectious centre assay was used to quantify both ecotropic and xenotropic virus; titres were expressed as $\log_{10}$ of the number of plaques or foci per 10^6 cells used in the assay. **a**, Ecotropic virus titres in 10% spleen extracts. In this assay, 2×10^5 SC-1 cells treated with $10 \mu\text{g ml}^{-1}$ polybrene were plated in 60-mm plastic Petri dishes and maintained in Eagle's minimum essential medium supplemented with 2 mM L-glutamine, antibiotics (25 U penicillin, 25 mg streptomycin per ml) and 5% fetal bovine serum. Twenty-four hours later plates were infected with 0.2 ml of the spleen suspensions and 2 d later the medium was changed. After 5 d the infected SC-1 cells were UV-irradiated ($20,000 \text{ erg mm}^{-2}$ for 30 s) and overlaid with 1×10^6 XC cells. Four days later, the cultures were fixed and stained, and the syncytial plaques were counted with an inverted microscope at $4\times$. Black circles represent virus titres of AKR/J(+/?), and open circles nu^{str}/nu^{str} . **b**, Ecotropic virus was assayed for by the infectious centre assay on SC-1 cells. The cells were infected with serial dilutions of washed lymphoid cells. After 5 d the infected plates were UV-irradiated and overlaid with XC-cells¹². Four days later the syncytial plaques were counted. Solid columns represent the virus titres of nu^{str}/nu^{str} spleen, open columns those of AKR/J spleen, and cross-hatched columns those of AKR/J thymuses. **c**, Xenotropic virus was detected by the infectious centre assay on the S+L- mink lung cells¹⁶. The S+L- cells were pretreated with polybrene as above and maintained in Dulbecco's medium supplemented with antibiotics and 10% heat-inactivated fetal bovine serum. Transformed cell foci produced by the rescued MSV pseudotypes were counted 8–10 d after infection.

thymus or a particular thymic cell population is not only necessary for leukaemogenesis but also for the isolation of MCF virus in AKR mice.

We compared ecotropic virus titres of starker mice with those of their normal littermates by the XC plaque assay using a cell line established from feral mice (SC-1)^{13,14}. Since we made

no attempts to distinguish by genetic test between the phenotypically normal heterozygote ($nu^{str}/+$) and homozygous wild-type mice ($+/+$), these normal mice will be designated as $+/?$. In a preliminary study involving few mice it was thought that ecotropic titres (using 10% tail suspensions) were significantly higher in starker mice³ than in normal AKR/J mice. However, as shown in Fig. 1a and b ecotropic virus was readily detected in tissues from both starker mice and heterozygotes. Also, we find no significant difference in ecotropic virus titre between the two phenotypes at the ages tested. To confirm that the ecotropic MuLV isolated from mutants was of the same tropism as AKR virus (FV-1ⁿⁿ), we infected SWR/J (Fv-1ⁿⁿ) and BALB/cJ (Fv-1^{bb}) cells at various multiplicities of infection. As expected the N-type SWR/J cells showed a level of sensitivity to virus infection approximately 100-fold greater than BALB/cJ cells¹⁵. These findings demonstrate that the mutant gene does not influence the expression of ecotropic virus. Also, as shown by others^{12,16}, the high incidence of leukaemia in AKR/J mice is not due solely to the persistently high titres of ecotropic virus.

The focus induction assay using Moloney transformed non-producer mink lung cells was used to detect xenotropic virus expression¹⁷. Low titres of virus were detected in spleens and thymuses of 3-month-old $+/?$ mice and their titres increased with age (Fig. 1c). By 5 months of age the thymuses had significantly higher titres of virus ($P \leq 0.01$ Student's *t* test) than did spleens from the same mice, whereas in starker mice low levels of xenotropic virus, comparable to that found in 3-month-old $+/?$ mice were detected in spleen cells at a later age (5 months). At 5 months the expression of xenotropic virus was significantly greater ($P \leq 0.01$) in tissues of $+/?$ mice compared to nu^{str} . By 8 months the virus titres in the spleens of $+/?$ and nu^{str}/nu^{str} were comparable, whereas $+/?$ thymus expressed a significantly higher titre when compared with the spleens of mice of either genotypes.

Table 1 Detection of endogenous MuLVs in nu^{str}/nu^{str} mice

Tissue	Age (months)	Infectious virus units per 10^6 cells ($\log_{10}$)		
		Ecotropic	Xenotropic	MCF
Leukaemic*	12	3.1	0.8	0
RCS†	8	3.0	0.7	0
AKR/J nu^{str}/nu^{str} (spleen)	8	3.0	0.8	0
AKR/J (spleen) $+/?$	8	2.5	2.2	ND‡

* Nine-month-old nu^{str}/nu^{str} mouse, grafted with 3-week-old AKR/J thymus developed leukaemia within 3 months.

† RCS in nu^{str}/nu^{str} .

‡ Not determined.

We analysed further the virus grown on mink lung cells for the presence of dual-tropic (MCF) viruses. Filtered supernatant fluids (Millipore, $45 \mu\text{m}$) from virus positive mink lung cells were transmitted to fresh cultures by limiting dilution technique⁸. Only supernatant fluid from cultures cocultivated with thymocytes of 8-month-old $+/?$ mice yielded a virus which induced morphological changes on mink lung cells and replicated in SC-1 cells as detected by the reverse transcriptase assay. One explanation for the absence of MCF virus in starker mice may be related to the lower titre of xenotropic virus thus decreasing the chance for recombination. A correlation between increased xenotropic virus expression and isolation of recombinant virus in preleukaemic mice and the subsequent development of spontaneous lymphoma has been established for another hairless mutant (*hr/hr*) of the HRS/J strain¹⁸.

We also assayed for recombinant viruses in several tissues from starker mice with reticulum cell sarcomas. Both eco- and xenotropic viruses were found in these tumours but no

virus(es)-induced morphological changes were seen on mink lung cells nor was a dual-tropic virus isolated (Table 1). These findings suggest that the thymus and/or high virus titres are necessary for the isolation of recombinant viruses. In preliminary transplantation studies, we found that 9-month-old streaker mice given thymic transplants from 3-week-old AKR/J mice, develop lymphatic leukaemia before 1 yr of age. Whereas low titres of both eco- and xenotropic viruses were detected, we have not isolated an MCF-like virus. Although a high proportion of leukaemic AKR/J mice express MCF virus it was not possible to isolate the virus from all such animals^{8,12}. Our failure to isolate an MCF virus may be due to the lower xenotropic virus titres (Table 1) in *nu^{str}* mice which received transplants, or perhaps because not enough mice were examined.

If high titres of both eco- and xenotropic viruses are necessary for recombination, then MCF-like virus should be expressed in tumours of the reticuloendothelial system other than thymomas. This hypothesis is supported by our findings of an MCF-like virus in a recombinant inbred line (BXH-2) which has a 100% tumour incidence (monocytic leukaemia) by 7–9 months of age (manuscript in preparation). The shortened lifespan of streaker mice compared with normal littermates (236.3 ± 13.61 and 307.4 ± 16.53 days respectively) may contribute to the low incidence of tumours observed in the total population of streaker mice. By increasing the lifespan of these mice (for example by raising them in germ-free conditions) we may increase their tumour incidence. Moreover, by examining more neoplasms as they occur we might be able to isolate an MCF-like virus from these mice.

Our results show no difference in the level of expression of ecotropic virus between streaker mice and littermate controls. However, beginning at 5 months of age there is a significant difference in xenotropic virus expression between both genotypes. The evidence suggests that recombinant viruses are generated independently, but the possibility of recombination occurring in an earlier generation and being preserved at a distinct genetic locus cannot be ruled out. More extensive thymic transplantation studies involving streaker mice may clarify some of these questions. Because the spontaneous remutation at the nude locus occurred in the inbred AKR/J strain, we can now exclude the possibility that the observed effects are due to one or more gene(s) closely linked to the nude locus rather than *nu* itself. The streaker mutation on the AKR/J background should provide a useful model for study of host-virus relationships and tumour expression.

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Structural polymorphism of human DR antigens

DR ANTIGENS are polymorphic cell surface molecules whose expression is controlled by a locus closely linked or identical to the D locus of the major histocompatibility complex (MHC) of man (for reviews see refs 1, 2). They are functionally and structurally homologous to the murine Ia antigens determined by the I-E subregion of the MHC, a region which has been implicated in the genetic control of immune responses^{3,4}. Both sets of antigens are mainly expressed on cells associated with immune function (for reviews see refs 1, 2, 5), and are involved in mediating T-cell, B-cell and macrophage interactions required for the generation of immune responses^{6–9}. In addition, both consist of two non-covalently associated polypeptides, designated α and β , with molecular weights of 34,000 and 28,000, respectively¹⁰. The association of some DR antigens with increased susceptibility to certain diseases (for review see ref. 1) and the genetic restrictions imposed on cellular interactions by the HLA-D region^{9,11} may represent the effects of structural variability among DR antigens. The aim of the studies reported here was to examine the nature and degree of structural variation among DR antigens isolated from cultured lymphoid B cells with different DR phenotypes. Such information may provide an understanding of the molecular mechanisms by which DR antigens mediate their function.

Human DR antigens labelled with ³H-phenylalanine were isolated from the cultured B-lymphoid cells Victor (DRw4, 6) and WiL-2 (DRw4, 7) by immunoprecipitation with the xenoantiserum 3634 as described elsewhere¹⁰. Purification of DR α and DR β chains was achieved by SDS polyacrylamide gel electrophoresis in the absence of reducing agents, thus permitting baseline separation of the α - and β -chains¹⁰. Proteolytic digestion of α - and β -chains with trypsin-TPCK and peptide analysis by isoelectrofocusing were carried out as described for the murine I-E subregion antigens¹². As the cell lines used in these studies are heterozygous, at least two α - and β -chains are being analysed.

Comparison of the peptide maps reveal that the α -chains isolated from the two lymphoid cell lines are similar, with some minor differences (Fig. 1); however, the two β -chains differ strikingly from one another. These differences are highly reproducible. This observation is consistent with our previous report¹⁰ that the N-terminal amino acid sequence of the DR β chain isolated from Victor cells differs from that of the DR β chain (p29) isolated from RPMI 4625 cells by Springer *et al.*¹³. Furthermore, our data parallel those obtained with I-E antigens, the murine counterpart of human DR antigens; partial amino acid sequence and peptide map comparison of I-E subregion antigens isolated from two MHC congenic strains of mice reveal several differences between the β -chains and an absence of any detectable differences between the α -chains¹⁴. In contrast, Klareskog *et al.*¹⁵ compared the structures of DR antigens isolated from Daudi and Raji lymphoblastoid cell lines and found that structural differences were restricted to the α -chain; at the moment we are unable to reconcile our results with those of Klareskog *et al.*¹⁵.

Because serological differences are a reflection of structural differences, the high degree of structural variation between β -chains isolated from different cell lines suggest that the β -chain carries the serologically defined DR alloantigenic determinants. Recent data by Tosi *et al.*¹⁶ demonstrating preferential binding of DR β chains to DR alloantisera support this proposal. If DR alloantigenic determinants are indeed determined by the β -chain, then this chain must be encoded by a gene in the MHC as familial studies demonstrate linkage of DR specificities with the MHC (for review see ref. 1). The murine studies, localising the gene encoding the E β chain to the MHC¹⁴, support this contention. The small peptide differences seen in the α -chains

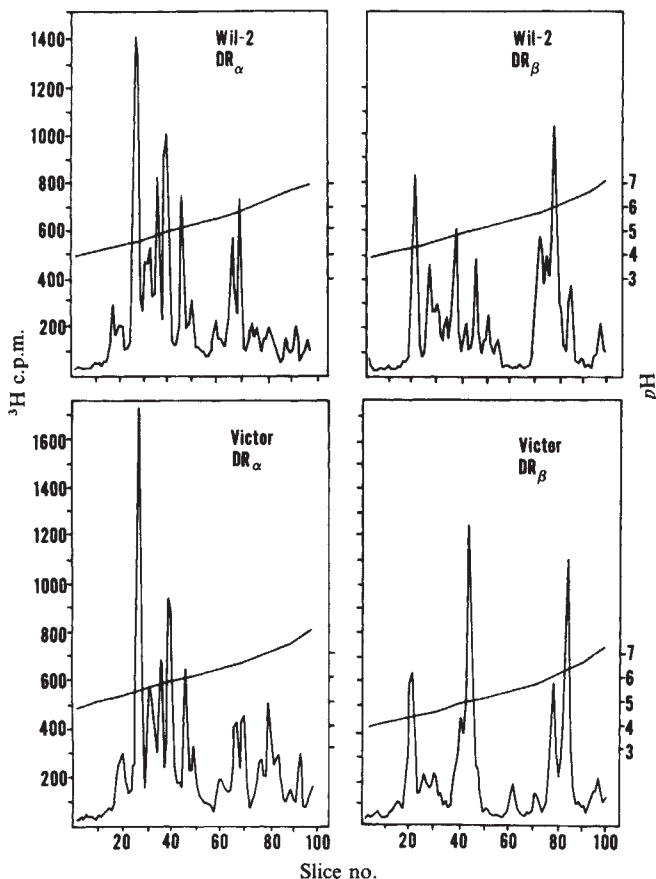


Fig. 1 Isoelectrofocusing gels of tryptic digests of DR α - and β -chains isolated from Victor and WiL-2 cells. DR antigens labelled with ^3H -phenylalanine were immunoprecipitated with xenoantiserum 3634. The α - and β -chains were subjected to proteolytic cleavage with trypsin-TPCK and the resultant component peptides were isoelectrofocused as described^{12,14}. The gel was then sliced into 1-mm sections and each slice was incubated with 0.7 ml water. After 3 h at 37 °C, the pH gradient was determined and each slice was counted in a liquid scintillation counter.

suggest some allelic variation of DR α chains but do not necessarily indicate genetic mapping in the MHC. Further structural and serological analysis, and immunogenetic studies with somatic cell hybrids, will clarify the molecular basis of serological polymorphism of DR antigens and define the genetic control of their expression.

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Structural polymorphism of I-E subregion antigens determined by a gene in the H-2K to I-B genetic interval

THE generation of immune responses in mice is influenced by Ir genes located in the I region of the major histocompatibility complex (MHC)¹. In some instances maximum responses require complementation by two genes, one in the I-A or I-B and the other in the I-E or I-C subregion^{2,3}. The effects of these genes are thought to be mediated by Ia alloantigens, which are cell surface molecules whose expression is controlled by the I region⁴. This is based on the observations that anti-Ia sera inhibit *in vitro* immune responses^{5,6}, and soluble factors that enhance *in vitro* immune responses express Ia alloantigenic determinants^{7,9}. Jones *et al.*¹⁰, using two-dimensional gel electrophoresis, observed that the expression of I-E subregion antigens is controlled by two genes, one in the I-A subregion, the other in the I-E subregion, and that the polymorphism of these antigens is influenced by an I-A subregion gene. As an explanation, the authors proposed that only one of the two polypeptide chains present in I-E immunoprecipitates is an I-E subregion product, the second being a product of the I-A subregion. Antisera obtained by cross-immunisation of I-E subregion-disparate strains of mice immunoprecipitates a molecular complex consisting of two chains, designated α and β , with molecular weights of 32,000 and 29,000 respectively¹¹⁻¹⁴. Previous studies suggested that I-E antigens isolated from B10.A(5R) and B10.D2 mice had identical α -chains but different β -chains¹⁵. However, as these mice differed at multiple genetic regions, it was not possible to show which I subregion(s) determined the polymorphism of the E_β chain. Therefore, we investigated the effects of the I-A subregion on the polymorphism of I-E subregion antigens. We have now shown by peptide mapping that the I-E subregion polymorphism which Jones *et al.* found to be controlled by the I-A subregion probably reflects structural polymorphism of β -chains controlled by an I-A subregion gene.

The structure of I-E subregion antigens isolated from three MHC congenic strains of mice, B10.A, B10.A(5R) and B10.S(9R), were compared. Two of these, B10.A(5R) and B10.S(9R) originated from recombination with B10.A; thus, all three strains have identical I-E subregions although they differ in the H-2K to I-B genetic interval (Table 1). I-E subregion antigens labelled with ^3H -phenylalanine were immunoprecipitated with antiserum (B10 $\times$ HTI) F_1 anti-B10.A(5R). Although this antiserum is potentially directed against antigens of multiple subregions to the right of the I-J subregion, serological analysis indicates that it reacts with an antigen determined by the I-E^k

Table 1 MHC genotypes of relevant mice

	Region or subregion								
	K	I-A	I-B	I-J	I-E	I-C	S	G	D
B10.A	k	k	k	k	k	d	d	d	d
B10.A(5R)	b	b	b	k	k	d	d	d	d
B10.S(9R)	s	s	s	k	k	d	d	d	d
(B10 $\times$ HTI) F_1	b/b	b/b	b/b	b/b	b/b	b/b	b/b	b/?	b/d

subregion¹⁶. I-E^k antigens isolated from B10.A, B10.A(5R) and B10.S(9R) mice and purified by SDS polyacrylamide gel electrophoresis in the absence of reducing agent displayed the α - and β -chains characteristic of such antigens¹². Both chains were subjected to proteolytic cleavage with trypsin-TPCK, and the resultant component peptides were examined by isoelectrofocusing, as described elsewhere^{15,17}. Figure 1 shows that α -chains from all three murine strains are virtually identical in peptide profiles, suggesting their structural identity. In contrast, the β -chains display one or more differences in their peptide profiles (Fig. 2). The identity of the three α -chain peptide profiles is evidence for the high degree of reproduction of the analytical method and strongly indicates that the differences observed in the β -chain peptide profiles do not represent artefacts. Thus, I-E^k antigens isolated from mice differing solely in the H-2K to I-B genetic interval have identical α -chains but structurally different β -chains. These results suggest that the I-A subregion-controlled polymorphism of I-E subregion antigens observed by Jones *et al.* reflect the structural polymorphism of β -chains controlled by an I-A subregion gene. Jones *et al.* also interpreted their observation as a possible explanation for the Ir gene complementation phenomenon.

Several models may be proposed to explain how two-gene control of I-E subregion antigens is related to Ir complementation. (1) As discussed by Jones *et al.*¹⁰ the α - and β -chains may

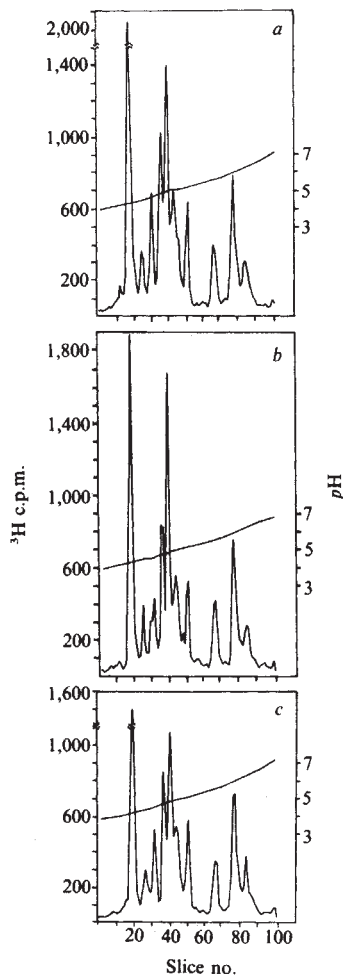


Fig. 1 Isoelectrofocusing gels of tryptic digests of E_α chains isolated from B10.A (a), B10.A(5R) (b) and B10.S(9R) (c) mice. I-E subregion antigens labelled with ^3H -phenylalanine were immunoprecipitated with antiserum (B10 $\times$ HTI) F_1 anti-B10.A(5R). E_α chains were subjected to proteolytic cleavage with trypsin-TPCK and the resultant component peptides were isoelectrofocused as described elsewhere^{15,17}. The gel was then sliced into 1-mm sections and each slice was incubated with 0.7 ml water. After 3 h at 37 °C, the pH gradient was determined and each slice was counted in a liquid scintillation counter.

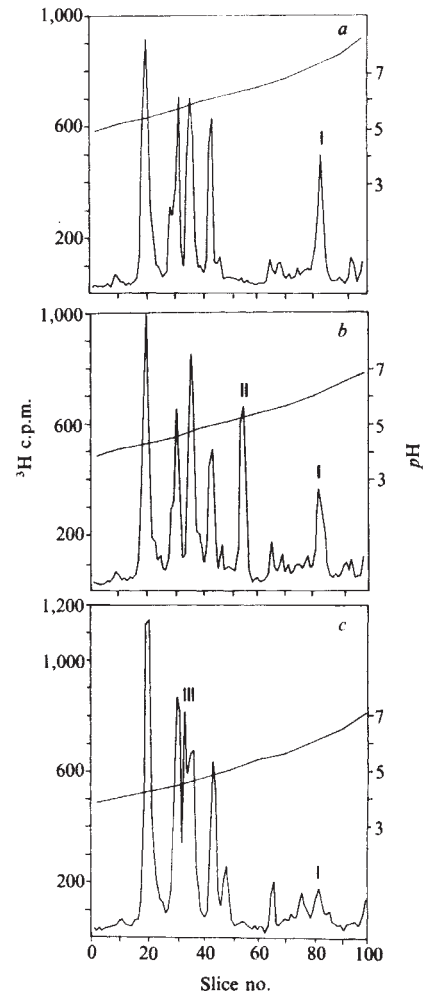


Fig. 2 Isoelectrofocusing gels of tryptic digests of ^3H -phenylalanine-labelled E_β chains isolated from B10.A (a), B10.A(5R) (b) and B10.S(9R) (c) mice. The peptide profiles were determined as described in Fig. 1. Peaks distinguishing peptide profiles from each other are designated I, II or III.

be encoded by genes with the I-E and I-A subregions, respectively, and only certain α - and β -chains may interact to form a functional molecule; (2) expression of E_α and E_β chains encoded by genes located outside the I-E subregion may be regulated by a gene located within the I-E subregion; (3) the gene product of the I-A subregion may modify the β -polypeptide after its biosynthesis.

Further genetic mapping of the component chains of Ia antigens and structural studies of their polymorphic polypeptides may distinguish between the alternative models of Ir gene complementation and increase our understanding of the relationship between Ia antigens and the development of immune responses.

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Evolutionary change in the insulin receptors of hystricomorph rodents

INSULIN receptors have been thought to have remained unaltered despite evolutionary changes in the hormone¹. Because insulins from hystricomorph rodents are known to be highly substituted compared with other mammalian insulins, we decided to investigate the insulin receptor of some hystricomorphs to determine whether evolutionary change had occurred within the receptor itself. Here we present the first evidence that hystricomorph rodent insulin receptors have undergone evolutionary change.

Hystricomorph rodents are a suborder of the Rodentia and contain over 180 species, including the coypu and the guinea pig². The insulins of these two animals are more substituted than those of any other mammal when compared with bovine or porcine insulin³. Study of hystricomorph rodent insulins could provide insight into the structure-activity relationships of insulin and may also throw some light on the evolution of this group of mammals. Chinchilla insulin has been sequenced in this laboratory⁴ and recently we sequenced casiragua (*Proechimys guairae*) insulin (Fig. 1). Both animals are hystricomorphs.

A chain

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
CASIRAGUA	GLY	ILE	VAL	ASP	GLN	CYS	CYS	THR	ASN	ILE	CYS	SER	ARG	ASN	GLN	LEU	LEU	THR	TYR	CYS	ASN	
COYPU	GLY	ILE	VAL	ASP	GLN	CYS	CYS	THR	ASN	ILE	CYS	SER	ARG	ASN	GLN	LEU	MET	SER	TYR	CYS	ASN	ASP
GUINEA PIG	GLY	ILE	VAL	ASP	GLN	CYS	CYS	THR	GLY	THR	CYS	THR	ARG	HIS	GLN	LEU	GLN	SER	TYR	CYS	ASN	
CHINCHILLA	GLY	ILE	VAL	ASP	GLN	CYS	CYS	THR	SER	ILE	CYS	THR	LEU	TYR	GLN	LEU	GLU	ASN	TYR	CYS	ASN	
BOVINE	GLY	ILE	VAL	GLU	GLN	CYS	CYS	ALA	SER	VAL	CYS	SER	LEU	TYR	GLN	LEU	GLU	ASN	TYR	CYS	ASN	

B chain

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
CASIRAGUA	TYR	VAL	GLY	GLN	ARG	LEU	CYS	GLY	SER	GLN	LEU	VAL	ASP	THR	LEU	TYR	SER	VAL	CYS	LYS	HIS	ARG	GLY	PHE		TYR	ARG	PRO	SER	GLU
COYPU	TYR	VAL	SER	GLN	ARG	LEU	CYS	GLY	SER	GLN	LEU	VAL	ASP	THR	LEU	TYR	SER	VAL	CYS	ARG	HIS	ARG	GLY	PHE		TYR	ARG	PRO	ASN	ASP
GUINEA PIG	PHE	VAL	SER	ARG	HIS	LEU	CYS	GLY	SER	ASN	LEU	VAL	GLU	THR	LEU	TYR	SER	VAL	CYS	GLN	ASP	ASP	GLY	PHE	PHE	TYR	ILE	PRO	LYS	ASP
CHINCHILLA	PHE	VAL	ASN	LYS	HIS	LEU	CYS	GLY	SER	HIS	LEU	VAL	ASP	ALA	LEU	TYR	LEU	VAL	CYS	GLY	ASP	ARG	GLY	PHE	PHE	TYR	THR	PRO	MET	ALA
BOVINE	PHE	VAL	ASN	GLN	HIS	LEU	CYS	GLY	SER	HIS	LEU	VAL	GLU	ALA	LEU	TYR	LEU	VAL	CYS	GLY	GLU	ARG	GLY	PHE	PHE	TYR	THR	PRO	LYS	ALA

Fig. 1 Comparison of the primary sequences of hystricomorph rodent insulins with bovine insulin.

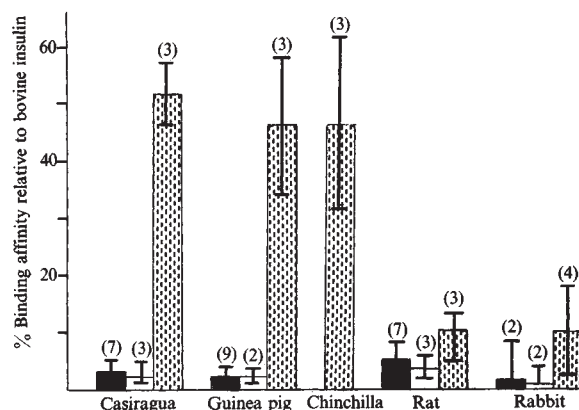


Fig. 2 The per cent binding affinity of casiragua, guinea pig and triphthaloyl bovine insulin on hystricomorph rodent, rat and rabbit liver plasma membranes. 95% Confidence limits are indicated. Stippled columns, casiragua insulin; open columns, guinea pig insulin; vertical-dashed columns, triphthaloyl bovine insulin. The figures in parentheses indicate the number of experiments used to derive the mean, with each experimental point determined from three replicates.

receptor is different from the insulin receptors of mammals such as the mouse, rat, rabbit and dog.

The data obtained with liver cell membranes suggest that the insulin receptors of the casiragua and chinchilla (Fig. 2) have changed in a similar manner to those of the guinea pig. Lack of animals prevented a more in-depth analysis of the *in vitro* and *in vivo* responses of the latter two animals to triphthaloyl bovine insulin. In all cases reported in the literature affinity data obtained from insulin binding to liver plasma membranes correlates with *in vitro* potency data obtained on isolated fat cells (for example see ref. 9). It seems reasonable to conclude, therefore, that the membrane binding potencies found in the casiragua and chinchilla would reflect the potency of triphthaloyl insulin on their fat cells.

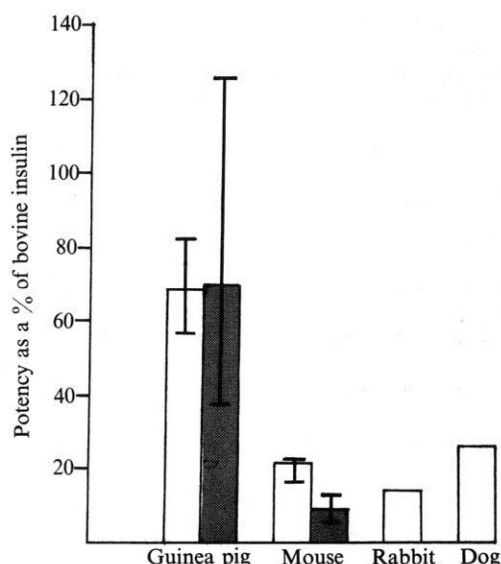


Fig. 3 Comparison of the potency of triphthaloyl bovine insulin with that in the mouse, rabbit and beagle dog. Potency fiducial limits ($P=0.95$) are indicated. Open columns, *in vivo* blood glucose lowering. Number of animals per dose and total used were respectively: guinea pig 7 and 35; mouse 24 and 96; rabbit 5-8 and 34; dog 2-4 and 20. Stippled columns, *in vitro* ^3H -glucose uptake into fat cells. The guinea pig and mouse fat cell potencies were obtained from the data from 6-7 and 6-9 experiments per dose respectively.

Interestingly, the insulin receptor in the chinchilla has undergone change despite the relatively few alterations in chinchilla insulin compared with bovine insulin. It might have been anticipated that the changes in the hystricomorph rodent insulin receptor demonstrated here, would have enabled hystricomorph rodent insulins to elicit both greater affinity for their own receptors and greater biological activity compared with bovine insulin. This is not the case. All hystricomorph rodent insulins are less potent than bovine insulin in the assay systems used here (Figs 2, 3). Indeed, they are less active than bovine insulin even when tested in hystricomorph rodents^{10,11}. Because bovine insulin is equipotent in assays using hystricomorph rodent or other mammalian tissues, it follows that the modified hystricomorph rodent insulin receptor retains intact the 'bovine type' insulin binding site. We have yet to determine whether the decreased biological activity of hystricomorph insulin (hypoglycaemia and stimulation of glucose uptake into lipid) is general for all biological activities or whether other activities, for example mitogenic, are conserved or enhanced.

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Short-latency drinking and increased Na appetite after intracerebral microinjections of NGF in rats

NERVE GROWTH FACTOR (NGF) is a polypeptide trophic factor for peripheral sympathetic and sensory neurones^{1,2}. Apparent NGF³⁻⁵ and NGF receptors^{6,7} have also been identified in the brain, and intracerebral administration of NGF in the adult rat produces marked biochemical⁸ and morphological^{9,10} changes in brain tissue. These findings, taken together with the observations that central injections of NGF facilitate behavioural recovery from brain damage^{11,12}, indicate that this polypeptide may have an important role in brain function. It has been observed that rats given intraventricular injections of up to 2.3 μg NGF drink copiously (M.E.L. and G. Guroff, unpublished observations). Perkins *et al.*¹³ reported that diencephalic

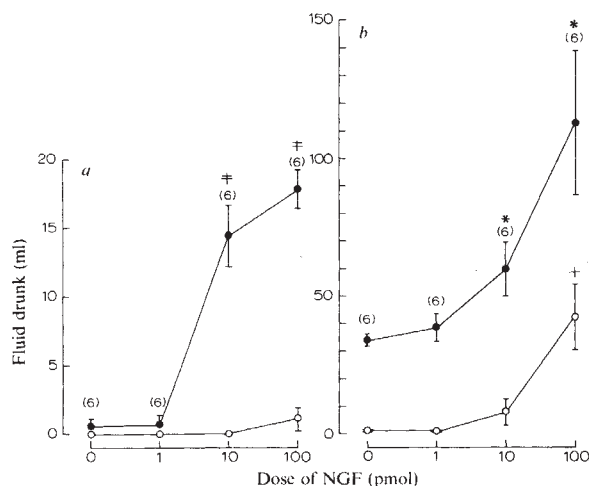


Fig. 1 The 1-h (a) and cumulative 18-h (b) intakes of water (●) and 2.7% NaCl (○) (mean values $\pm$ s.e.m., number of observations in parentheses) in rats after intracranial injections of various doses of NGF for the first time through cannulae aimed at the preoptic area. The rats were individually housed with free access to standard rat pellets and to water and 2.7% NaCl contained in graduated burettes. The rats were injected with 1–2 μ l of vehicle (0.05 M sodium acetate, pH 5) containing 2.5 S NGF (molecular weight 26,518, prepared by the method of Bocchini and Angeletti¹⁴). Controls received injections of vehicle alone. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

application of crystalline NGF (1–15 μ g) resulted in an intense polydipsia. The present report confirms the observations of M.E.L. and Guroff, and extends the findings of Perkins *et al.*¹³ by using solutions of NGF instead of crystals. It also describes for the first time a second phenomenon produced by intracranial administration of NGF, namely an intense appetite for aversive concentrations of sodium solutions.

Single intracranial microinjections of NGF caused vigorous drinking in rats that were in water balance (Fig. 1). All results in Fig. 1 were obtained with first injections in rats which had had no previous experience of intracranial injections of NGF or any other substance. Rats injected with 10 and 100 pmol NGF initially chose water after latencies of 455 ± 180 s and 212 ± 37 s, respectively, all rats in both groups responding. The animals did not seem to be disturbed by the injections and the drinking behaviour produced by NGF was entirely normal. With the

Table 1 The 1-h and 18-h cumulative intakes of water and NaCl after NGF administration

	n	1 h		18 h	
		Water (ml)	2.7% NaCl (ml)	Water (ml)	2.7% NaCl (ml)
a Control	4	0	0	40.75 $\pm$ 5.64	0.27 $\pm$ 0.08
NGF	5	18.64 $\pm$ 3.60†	2.96 $\pm$ 1.64	130.84 $\pm$ 29.95*	56.44 $\pm$ 19.99*
b Control	10	0	–	29.87 $\pm$ 2.41	–
NGF	5	17.60 $\pm$ 1.52‡	–	126.24 $\pm$ 26.44‡	–
c Control	14	–	1.23 $\pm$ 0.53	–	11.80 $\pm$ 2.17
NGF	5	–	2.08 $\pm$ 0.52	–	58.80 $\pm$ 5.12‡

The 1-h and 18-h cumulative intakes (mean $\pm$ s.e.m.) of: a, water and 2.7% NaCl when both solutions were available; b, when only water was available; c, when only 2.7% NaCl was available, after preoptic injection of 50 pmol NGF.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$, compared with corresponding controls.

doses used, drinking continued for the whole of the test period (18 h) and was dose dependent. Overnight food intake was considerably reduced, falling from 25.62 ± 1.87 g in controls to 9.62 ± 1.83 g ($n = 4$) after 50 pmol NGF.

The second behaviour, not previously reported, was an increase in sodium intake (Fig. 1). NGF (100 pmol) produced a small intake of 2.7% NaCl within 1 h of injection, and only three of six rats drank NaCl solution during this time (mean latency $1,340 \pm 322$ s). The main intake of 2.7% NaCl solution occurred overnight, with extremely large intakes ($P < 0.01$) following 100-pmol doses. The huge intakes of 2.7% NaCl following NGF administration reflect a specific appetite for sodium rather than a loss of discriminative ability, for when equimolar KCl was offered with 2.7% NaCl and water after 50 pmol NGF ($n = 6$), the cumulative 18-h intakes were: water, 93.80 ± 18.80 ml; 2.7% NaCl, 55.57 ± 15.00 ml; 3.45% KCl, 0.23 ± 0.06 ml.

Subsequent injections of NGF produced greater intakes of both water and 2.7% NaCl. For this reason, a further dose-response study was carried out on rats that had had at least two previous injections of NGF (Fig. 2). At 1 h after injection the intakes of 2.7% NaCl in the experienced rats after 10 and 100 pmol NGF were significantly greater ($P < 0.05$) than in the naive animals given the same doses of NGF. By 18 h after injection the cumulative intakes of water and 2.7% NaCl in experienced rats given 1 and 10 pmol NGF were significantly greater than in naive animals (1 pmol, $P < 0.05$; 10 pmol, $P < 0.01$). The control intake of 2.7% NaCl was also significantly greater in the experienced rats ($P < 0.05$). The other values for experienced and naive rats were not significantly different.

Table 2 The 1-h and 18-h cumulative intake (mean $\pm$ s.e.m.) of water and 2.7% NaCl, when both solutions were available, by nephrectomised rats after preoptic injection of 50 pmol NGF

	n	1 h		18 h	
		Water (ml)	2.7% NaCl (ml)	Water (ml)	2.7% NaCl (ml)
Control	7	1.07 $\pm$ 0.54	0	22.21 $\pm$ 3.46	0.31 $\pm$ 0.14
NGF	10	21.92 $\pm$ 2.76‡	2.24 $\pm$ 0.89	36.84 $\pm$ 4.22*	3.82 $\pm$ 0.88†

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$, compared with corresponding controls.

To determine to what extent thirst and sodium appetite were independent responses, additional groups of rats were allowed access to only one solution after injection of NGF (Table 1). Rats offered only water showed a striking and sustained polydipsia in which the intakes were significantly greater than those of controls at both 1 h and 18 h after injection. On the other hand, the intakes of water in the water-only group at 1 h and 18 h after injection were not significantly different from the corresponding intakes of water in the group that had had access to both water and 2.7% NaCl. Rats offered 2.7% NaCl only showed a small but insignificant increase in intake during the first hour, but a very large intake during the subsequent hours (Table 1). As in the water-only experiment, the 1-h and 18-h intakes of 2.7% NaCl were not significantly different from the intakes of 2.7% NaCl in the two-solution group. Thirst therefore precedes sodium appetite and there is little interaction between the two behaviours. Water intake is not necessary to generate a sodium appetite nor is the overnight water intake secondary to the sodium appetite.

The short latency of the water intake makes it unlikely that the polydipsia is secondary to an NGF-induced polyuria. The slower time course of the increase in sodium appetite, however, could have been secondary to an NGF-induced natriuresis. Rats injected with 50 pmol NGF 3 h after bilateral nephrectomy and allowed access to water and 2.7% NaCl showed a short latency (120 ± 19 s, $n = 6$) and vigorous water drinking response to NGF (Table 2). The reduction in 18-h water intake is certainly accounted for by the increasing positive fluid balance and dilutional inhibition as drinking proceeded.

There was also a significant increase in the intake of 2.7% NaCl by nephrectomised rats injected with 50 pmol NGF

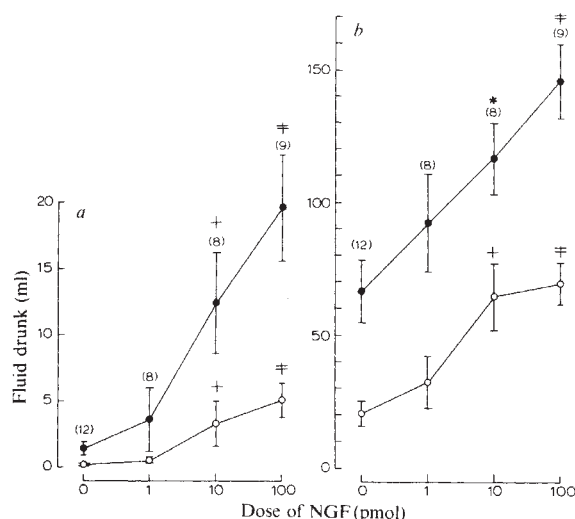


Fig. 2 The 1-h (a) and cumulative 18-h (b) intakes of water (●) and 2.7% NaCl (○) (mean values $\pm$ s.e.m., number of observations in parentheses) in rats after intracranial NGF administration. Methods were as in Fig. 1, except that the injections were made in rats that had received at least two injections of NGF previously. All injections were separated by an interval of 1 week. A few results on rats with cannulae in the third cerebral ventricle are included here as the results were the same as for preoptic injections. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

(Table 2), but intake was reduced to a greater degree than water intake after removal of the kidneys. This attenuation is due to increasing positive sodium balance ($1,764.8 \pm 406.6 \mu\text{mol}$, $n = 10$) and the reluctance of a uraemic animal to drink hypertonic solutions.

The primacy of NGF-stimulated sodium intake is further demonstrated by hourly sodium balance measurements in intact rats injected with 50 pmol NGF (Table 3). The rats remained in positive sodium balance throughout the period during which the intake of 2.7% NaCl was increasing. Natriuresis was therefore mainly the consequence and not the cause of the increased intake of 2.7% NaCl. Furthermore, the positive sodium balance of these animals was similar to that of the nephrectomised group.

These findings demonstrate that intracranial injections of minute quantities of NGF reliably elicit short-latency and dose-dependent drinking. In addition, NGF produces a delayed and dose-dependent sodium appetite. The intake of 2.7% NaCl in rats injected with NGF is huge, and considerably greater than that observed following adrenalectomy or any other stimulus to sodium appetite. The finding that both responses increase with repeated injections of NGF suggests that some powerful learning or sensitisation process is taking place.

There was a marked similarity between the effects of intracranial injections of NGF and of renin (D.B.A. and J.T.F., unpublished). Unlike angiotensin II, which produces an immediate thirst, renin and NGF stimulate water intake after a

delay of several minutes. In addition, the initial water intake following either renin or NGF is followed by a sustained and massive intake of 2.7% NaCl. It remains to be determined whether the thirst and sodium appetite elicited by NGF is mediated by the cerebral isorenin-angiotensin system or by a still unidentified neural system involved in the behavioural regulation of fluid and electrolyte balance.

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High cell density alters the ratio of type III to I collagen synthesis by fibroblasts

HUMAN fibroblasts in culture synthesise both type I and type III collagen¹, with type I accounting for 70–90% of the total². In culture, the rates at which these proteins are synthesised are constant and apparently rather rigidly controlled³. However, the proportions of these collagens differs in cells cultured with increased amounts of serum (increased type III/I)⁴ as well as in cells obtained from patients with certain diseases. Cells from patients with the Ehlers-Danlos type IV syndrome make little or no type III collagen^{5,6}, whereas cells from patients with osteogenesis imperfecta have an increased type III/I (refs 7, 8). We have found that cells from some patients with systemic sclerosis (scleroderma), have a reduced type III/I ratio. However, as previously reported, these cells grew to a lower density than control cells⁹. We report here that normal fibroblasts from human and guinea pig skin produce proportionally more type III collagen at high cell density, probably because of a reduction in the synthesis of type I collagen.

Table 1 Proline incorporation into proteins and collagen fractions from cultured guinea pig fibroblasts

	High density (8×10^6 cells per 75 cm^2)	Low density (8×10^6 cells per 300 cm^2)
Total non-dialysable radioactivity in media	29.8	43.7
Type I collagen (+)		
type I procollagen	1.0	3.1
Type III procollagen	0.7	1.3

Results expressed as d.p.m. $\times 10^{-6}$ from pooled area indicated in Fig. 3.

Table 3 The cumulative hourly intake of water and the intake, output and balance of sodium (mean values $\pm$ s.e.m.) after NGF administration

Hour	Water intake (ml)	Na intake (μmol)	Na output (μmol)	Na balance (μmol)
1	13.40 ± 1.75	$1,016 \pm 463$	455 ± 53	$+561 \pm 465$
2	14.80 ± 1.90	$2,476 \pm 868$	$1,243 \pm 256$	$+1,233 \pm 638$
3	19.20 ± 3.80	$4,213 \pm 1,218$	$2,305 \pm 615$	$+1,908 \pm 660$
4	27.52 ± 4.53	$7,244 \pm 1,669$	$3,912 \pm 949$	$+3,331 \pm 884$
5	31.56 ± 5.35	$8,907 \pm 2,237$	$6,227 \pm 1,528$	$+2,679 \pm 919$
6	39.44 ± 6.65	$11,531 \pm 2,472$	$8,516 \pm 2,058$	$+3,014 \pm 696$
7	48.92 ± 8.24	$14,063 \pm 2,898$	$11,098 \pm 2,402$	$+2,955 \pm 638$
8	59.88 ± 9.04	$16,963 \pm 3,490$	$13,433 \pm 2,978$	$+2,301 \pm 903$

Results are from five rats offered water and 2.7% NaCl (1 ml = $462 \mu\text{mol}$ Na) after preoptic injection of 50 pmol NGF. Cumulative intake of 2.7% NaCl at 8 h was $36.72 \pm 7.55 \text{ ml}$, mean sodium balance remained significantly positive ($P < 0.05$) throughout the experiment.

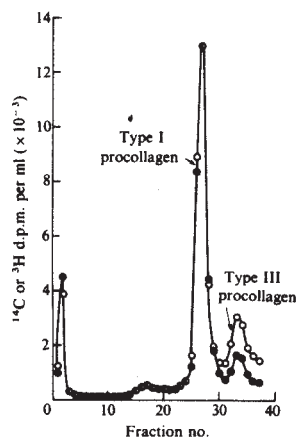


Fig. 1 DEAE-cellulose column chromatography of types I and III collagenous proteins in culture media from equal numbers of human skin fibroblasts plated at medium (●) and high (○) density. Cells (3×10^5) were plated on to tissue culture dishes of different size (75 or 25 cm²). After 24 h the media were changed to 10 ml of serum-free Dulbecco-Vogt media supplemented with ascorbic acid (100 μ g ml⁻¹) and β -aminopropionitrile (β -APN) (100 μ g ml⁻¹), and ¹⁴C-proline or [2,3-³H]proline for medium-density or high-density cultures, respectively (5 μ Ci ml⁻¹). Collagenous proteins were isolated from the media by precipitation with (NH₄)₂SO₄ (1.14 g per 10 ml) in the presence of protease inhibitors (20 mM EDTA, 1 mM *p*-hydroxymercuribenzoate PMB and 10 μ M PMSF). Collagenous proteins were redissolved and dialysed against 50 mM Tris-HCl (pH 7.4), containing 2 M urea (starting buffer). DEAE-cellulose column (1.6 $\times$ 4 cm) chromatography was carried out with a 0–0.2 M NaCl linear gradient in starting buffer, over a total volume of 400 ml at 4°C. Fractions of 10 ml were collected and the radioactivity of aliquots was determined with a Beckman liquid scintillation system LS-335 in 10 ml of Hydromix (Yorktown Research).

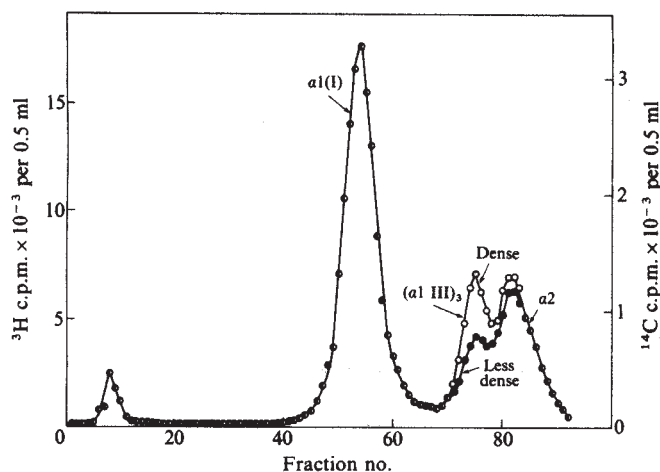


Fig. 2 CM-cellulose column chromatography of pepsin-treated collagenous proteins from media and from cell layers of human skin fibroblasts plated at medium and high density. Cells, 3×10^6 (medium) and 6×10^6 (high), were plated on to tissue culture flasks (75 cm²). After 24 h the media were changed to 10 ml of serum- and glutamine-free Dulbecco-Vogt medium supplemented with ascorbic acid (100 μ g ml⁻¹) and β -APN (100 μ g ml⁻¹), and [2,3-³H]proline or ¹⁴C-proline for high-density (○) or medium-density (●) cultures, respectively (5 μ Ci ml⁻¹). After 24 h, media and cell layers from both cultures were collected, combined, supplemented with 10 mg of carrier collagen from lathyritic-rat skin and treated with pepsin for 6 h at 15°C. CM-cellulose column (1.6 $\times$ 3 cm) chromatography was carried out in 4 M urea and 40 mM sodium acetate buffer, pH 4.8. A 0–90 mM NaCl linear gradient was used over a total volume of 200 ml at 45°C. Fractions of 2.5 ml were collected and the radioactivity of aliquots determined.

Human skin fibroblasts were obtained from American Type Culture Collection, Rockville, and guinea pig cells from primary culture of guinea pig dermis. Cultures with different cell densities were either selected from cultures started with different numbers or prepared by plating equal numbers of cells in tissue culture flasks of different sizes. In the latter case, approximately 3×10^6 human skin fibroblasts were plated on to 150, 75 or 25 cm² culture flasks and 8×10^6 guinea pig skin fibroblasts were plated on to a total area of 300 or 75 cm² in culture flasks.

The ratio of the collagens synthesised by human cells was estimated by DEAE-cellulose column chromatography and by CM-cellulose column chromatography after pepsin treatment. Types I and III collagen were synthesised in the same proportions by human cells at low and at moderate density (not shown). However, at high density proportionally more type III collagen was produced. Similar results were obtained with both procedures for separating the collagens (Figs 1, 2). The conversion of procollagen type I to collagen type I was greater at high density, but this does not seem to be important here, because

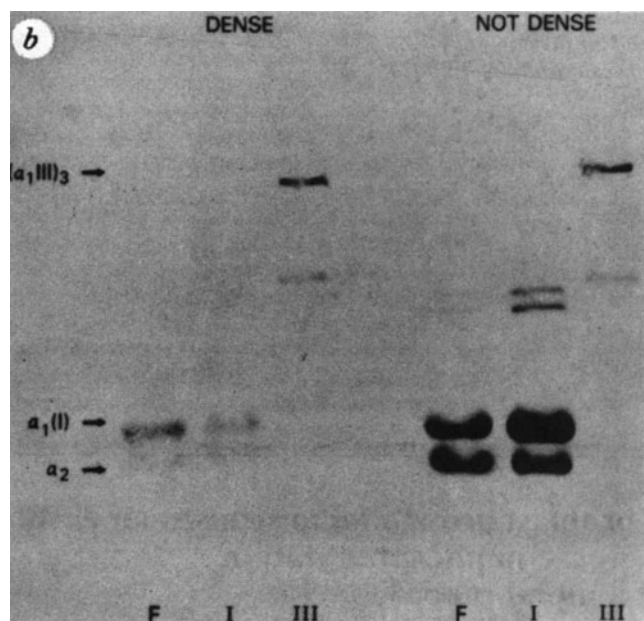
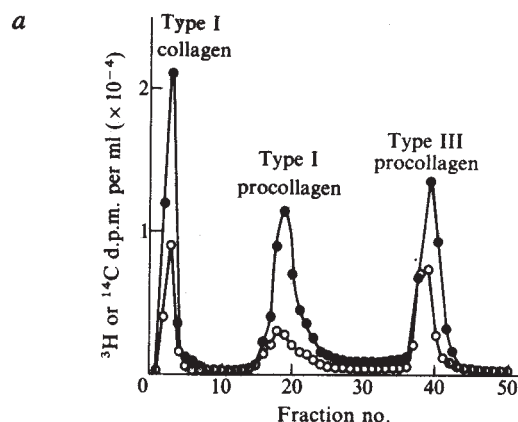


Fig. 3 DEAE-cellulose column chromatography of types I and III collagenous proteins of culture media from equal numbers of guinea pig skin fibroblasts plated at medium and high density. *a*, Cells (8×10^6) were plated on to tissue culture dishes of different size (75 or 300 cm²). The culture, labelling and column conditions were the same as in Fig. 1, except that the volume of culture media was 24 ml. ○, High density; ●, low density. *b*, Aliquots of material in the front (tube nos 2–6) as well as peaks of procollagen type I (tube nos 16–22) and of procollagen type III (tube nos 36–42) were treated with pepsin and then electrophoresed. The positions for $\alpha 1(III)_3$, $\alpha 1(I)$ and $\alpha 2$ are indicated, F indicates front, I is from procollagen type I peak and III from procollagen type III peak.

identical results were observed when collagens were solubilised with pepsin and their components resolved by CM-cellulose column chromatography (Fig. 2). Only 10–20% of the total hydroxyproline was associated with the cell layer at all cell densities.

To confirm the influence of cell density on collagen synthesis in fibroblasts of different species, we plated an equal number of guinea pig cells in dishes of different size and labelled them with ^3H - or ^{14}C -proline for 24 h. Total protein synthesis in the cells at high density was 70% of that observed in the cells at low density (Table 1). The collagenous proteins produced and secreted into the media were separated by DEAE-cellulose column chromatography (Fig. 3a), and the peaks identified by SDS-gel electrophoresis of an aliquot which was digested with pepsin (Fig. 3b). The ratio of $[\alpha 1(\text{III})]_3$ to $\alpha 1(\text{I})$ was 0.42 and 0.7 at low and high densities, respectively. This difference in ratio was due to a reduction of collagen synthesis in cells at high density, the synthesis of type I collagen being reduced more than that of type III (Table 1).

Our studies show that at high cell density cultured fibroblasts synthesise an increased ratio of type III/I collagen. The absence of this finding in fetal lung cell studies³ may have been due to the fact that the cells were less affected by crowding. As the synthesis of these collagens is altered in certain genetic conditions, it may be necessary to study cells at more than one density to establish their collagen phenotype. The changes observed could be caused by changes in collagen synthesis or degradation¹⁰. However, because recent studies show that elevations in cyclic AMP reduce collagen production¹¹, it is possible that the density-dependent changes observed here reflect the increase in cyclic AMP levels known to occur in cells at high density.

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Fibroblast growth factor causes an early increase in phosphorylation of a membrane protein in quiescent 3T3 cells

THE regulatory systems controlling cell division have not been identified, but it has been shown that growth factors such as epidermal growth factor, fibroblast growth factor (FGF), and serum initiate rapid changes in cellular metabolism, probably involving post-transcriptional control mechanisms^{1–5}. As phosphorylation has been shown to be an important regulatory mechanism in several metabolic pathways, we initiated experiments to determine whether factors which stimulate DNA synthesis also stimulate endogenous phosphorylation. We find that, within 5 min of addition of FGF or serum to ^{32}P -labelled

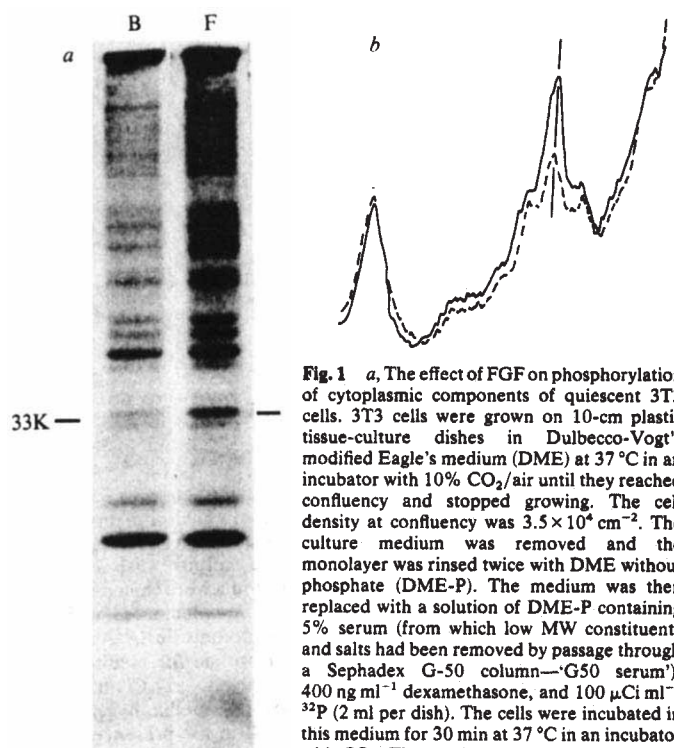


Fig. 1 a, The effect of FGF on phosphorylation of cytoplasmic components of quiescent 3T3 cells. 3T3 cells were grown on 10-cm plastic tissue-culture dishes in Dulbecco-Vogt's modified Eagle's medium (DME) at 37 °C in an incubator with 10% CO_2 /air until they reached confluency and stopped growing. The cell density at confluency was $3.5 \times 10^6 \text{ cm}^{-2}$. The culture medium was removed and the monolayer was rinsed twice with DME without phosphate (DME-P). The medium was then replaced with a solution of DME-P containing 5% serum (from which low MW constituents and salts had been removed by passage through a Sephadex G-50 column—G50 serum), 400 ng ml^{-1} dexamethasone, and 100 $\mu\text{Ci ml}^{-1}$ ^{32}P (2 ml per dish). The cells were incubated in this medium for 30 min at 37 °C in an incubator with CO_2 . The medium was then removed, the cell monolayer rinsed twice with DME-P, and the original culture medium—to which was added 400 ng ml^{-1} dexamethasone and 0.005% bovine serum albumin (BSA), with or without 50 ng ml^{-1} bovine pituitary FGF—was placed over the cells (4 ml per dish). The cells were incubated at 37 °C for 5 min, and then the medium was removed; the cells were rinsed twice with buffer (0.14 M NaCl, 6.7 mM KCl, 0.68 mM CaCl_2 , 0.49 mM MgCl_2 , 0.37 mM Na_2HPO_4 , 25 mM Tris, pH 7.4), and then the dishes were placed on a bed of ice and the cells were scraped into 0.5% NP40 plus 1 mM CuSO_4 at 4 °C (0.5 ml per dish). The cells were homogenised by drawing the suspension up and down, 10 times, through a Pasteur pipette. The nuclei were removed by centrifugation at 2,500g for 15 min. The supernatant was removed and diluted twofold with gel buffer to a final 2% SDS, 2% β -mercaptoethanol, 10% glycerol, 0.004% bromophenol blue, 59 mM Tris- PO_4 , pH 6.9. Samples were heated at 98 °C for 2 min before resolution by SDS gel electrophoresis according to Laemmli¹⁹ with the modification that the concentration of Tris-HCl in the running-gel buffer was 0.6 M pH 8.9. The acrylamide concentration in the gel was in the form of a gradient of 7.5% to 15% (top to bottom). Thirty μl of each sample was applied per channel. The 33,000-MW protein is indicated (33K). B, BSA; F, FGF. b, Densitometric scans of the region around the 33,000-MW component on the autoradiograms shown in a. The position of the 33,000-MW component on the scan of the BSA control (---) and FGF stimulated (—) cell post-nuclear supernatants is indicated by the vertical line. The effect of FGF in this experiment was to increase the area under the peak at 33,000-MW, relative to the reference peak indicated by the dot, to 172% of the BSA control.

Swiss 3T3 cells, there is a specific increase in the phosphorylation of a membrane protein with an apparent molecular weight of 33,000. Experiments with isolated cell fractions demonstrate that the phosphorylation of this protein is stimulated by cyclic AMP. This rapid and specific response to mitogens raises the possibility that this phosphorylation might be part of the initial, cellular signal for DNA synthesis.

Differences in cyclic AMP-stimulated phosphorylation patterns of cytoplasmic proteins derived from quiescent and growing 3T3 cells have been reported⁶. In those studies cytoplasmic extracts were phosphorylated by addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Thus, cellular compartmentalisation had been destroyed and the resulting differences in phosphorylation patterns do not necessarily reflect what is occurring in the intact cell. Because of this consideration, we did our experiments with intact ^{32}P -labelled cells.

Quiescent 3T3 cells were labelled with ^{32}P for 0.5 to 1 h. (Incorporation of ^{32}P into trichloroacetic acid-precipitable material was linear for 6 h.) The cells were prelabelled with ^{32}P , then incubated in medium with or without the mitogen in the

absence of external ^{32}P , to avoid the effect on ^{32}P incorporation of an increase in the specific activity of phosphate pools caused by an early stimulation of phosphate uptake by mitogens^{4,5}.

Five minutes after the addition of FGF to quiescent, ^{32}P -labelled 3T3 cells, we often observed an increase in phosphorylation, above background, of many components in the post-nuclear supernatant (Fig. 1). Most pronounced and always observed was an increase in phosphorylation of a component of apparent molecular weight $33,000 \pm 400$. The area under the peak of the 33,000-MW protein was determined from densitometric scans of autoradiograms in this and two other experiments, and normalised to the area under another phosphorylated peak. Compared with the bovine serum albumin (BSA) control, FGF stimulated phosphorylation of the 33,000-MW protein by $151 \pm 15\%$ (s.d.) of the control ($n = 4$). The effect of FGF on phosphorylation is observed as early as 5 min after addition of the mitogen and it persists for at least 60 min. We have tested the effect of three different mitogens for 3T3 cells on the phosphorylation patterns of ^{32}P -labelled quiescent 3T3 cells—calf serum, FGF from pituitary⁷ and FGF from brain⁸. All three mitogens stimulate the phosphorylation of the 33,000-MW component.

The subcellular localisation of the increased phosphorylation was investigated. Cells labelled with ^{32}P were incubated for 20 min with either BSA, FGF with BSA, or serum, and then fractionated according to the scheme outlined in Fig. 2. In the fraction (DP), that consists mainly of membrane vesicles from the endoplasmic reticulum and plasma membrane⁹, both FGF and serum cause a pronounced increase in phosphorylation of the 33,000-MW protein. From densitometric scans of autoradiograms from two experiments, the stimulation relative to the BSA control (normalised to another phosphorylated

protein on the same autoradiogram) was 214% for FGF and 282% for serum. The 33,000-MW component in fractions designated CP and DS is not phosphorylated to any greater degree than the general increase in phosphorylation of components in these fractions.

In the nuclear fraction, BP, a response to FGF in phosphorylation of the 33,000-MW component can be detected, although this is minimal and is not always observed. As well as nuclei, this fraction contains some unbroken cells, which probably contributes to its enzymatic-marker activity for plasma membrane (11% of the total recoverable 5'-nucleotidase). Increases in phosphorylation of nuclear proteins have been observed in other cell types within an hour after addition of serum or a peptide hormone^{10,11}. We find no reproducible early response (up to 20 min) to FGF on phosphorylation in 3T3 nuclei prepared by solubilisation of the cells in NP40 as described in Fig. 1 legend. Hence, the major portion of the response to these two mitogens on phosphorylation of the 33,000-MW component is located in a fraction enriched in enzymatic markers for the plasma membrane and endoplasmic reticulum (49% of the total recoverable 5'-nucleotidase, 30% of the total recoverable NADH oxidase).

To determine the nature of the acceptor for ^{32}P , we treated the cytoplasmic extract of ^{32}P -labelled, 3T3 cells, to which FGF had been added, with pronase or trypsin. Such treatment results in the disappearance from the gels of all phosphorylated bands. Protease digestion results in the solubilisation of 44% (pronase) and 42% (trypsin) of the incorporated trichloroacetic acid-precipitable radioactivity. Neither deoxyribonuclease I nor ribonuclease, both at $50 \mu\text{g ml}^{-1}$, have a significant effect on the incorporated trichloroacetic acid-precipitable counts, relative to the control, after 2 h incubation at 37°C . In the same conditions,

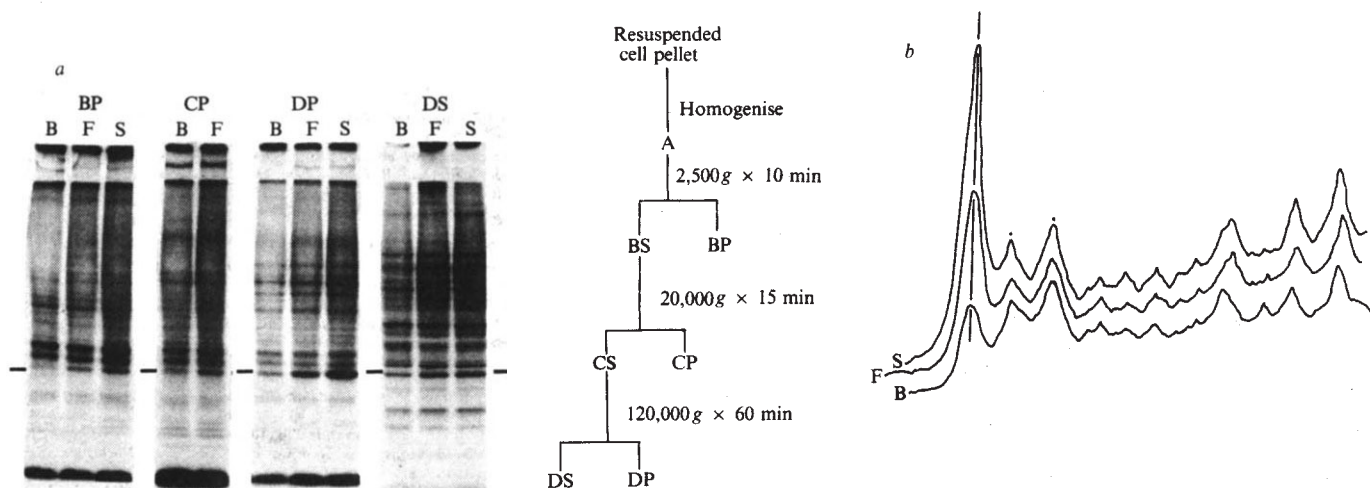


Fig. 2 *a*, Subcellular localisation of the phosphoprotein whose phosphorylation is stimulated by FGF. The medium over quiescent 3T3 cells was changed to fresh DME containing 10% of the normal phosphate content, 2% calf serum, and 400 ng ml^{-1} dexamethasone. After 17 h at 37°C in an atmosphere of 10% $\text{CO}_2/90\%$ air, two-thirds of the medium was removed and ^{32}P was added to the remaining medium to a final concentration of $300 \mu\text{Ci ml}^{-1}$. The cells were incubated in this medium for another hour. The labelling medium was removed and replaced by the original two-thirds of the conditioned culture medium to which had been added either 0.005% BSA (B), 0.005% BSA and 50 ng ml^{-1} brain FGF (F), or 10% serum (S). The cells were incubated at 37°C with 10% $\text{CO}_2/90\%$ air for 20 min. The cell monolayer was rinsed twice with buffer as in Fig. 1. Then the cells were scraped from the dish with a rubber blade into 0.15 M NaCl , 0.04 M NaF , 0.01 M KPi (pH 7.3) at 4°C and fractionated according to the method of Wallach and Kamat²⁰, modified as described previously²¹. Sodium fluoride (40 mM) was included in all buffers to reduce phosphatase action. The cellular fractions were then resuspended and their protein concentrations determined by intrinsic protein fluorescence²². Samples for electrophoresis were immediately diluted with gel buffer and boiled as described in Fig. 1. DS samples were concentrated by trichloroacetic acid precipitation before dissolving in gel buffer. For each cellular fraction, the samples corresponding to the three treatments were adjusted for protein concentration and equal amounts of protein were applied to each of the three channels on the SDS 7.5%–15% acrylamide gradient slab gels. FGF was isolated from bovine brain according to the procedure of Gospodarowicz *et al.*⁸. The membrane preparation procedure took 2 h to complete and was carried out at 4°C . The possibility that the specific increase in phosphorylation of the 33,000-MW protein is an apparent increase resulting from differential phosphatase action over the 2-h preparation period is unlikely for the following reasons: (1) When incubated for 2 h at 37°C in the presence of 40 mM NaF only 29% of the total trichloroacetic acid-precipitable ^{32}P counts are lost from a postnuclear-supernatant preparation. This translates to a loss of 4% in 2 h at 4°C (using a Q_{10} of 2 for phosphatase activity). (2) When membrane samples, phosphorylated *in vitro*, are incubated at 37°C , the 33,000-MW protein is dephosphorylated more rapidly than other membrane proteins. This would decrease rather than increase the observed stimulation of phosphorylation of the 33,000-MW protein. An alternative possibility, that differential protein kinase activity during the preparation could lead to the observed specific increase in phosphorylation is unlikely for several reasons: (1) The cell pellet was diluted to ~50 times its volume when homogenised, resulting in a 50-fold decrease in intracellular ATP concentrations. And (2) the preparation was done in the presence of low (0.2 mM MgCl_2), insufficient for activation of cellular protein kinases which are very Mg dependent, requiring 10–20 mM for optimal activity. *b*, Densitometric scans of the region around the 33,000-MW component on the autoradiograms of DP shown in *a*. The position of the 33,000-MW component on the scan of the BSA control (B), the FGF-stimulated (F) and serum-stimulated (S) membrane fractions is indicated by the vertical line. The effect of the mitogens in this experiment was to increase the area under the peak at MW 33,000, relative to the area under two adjacent phosphorylated peaks (indicated by the dots) to 180% (FGF) and 282% (serum) of the BSA control.

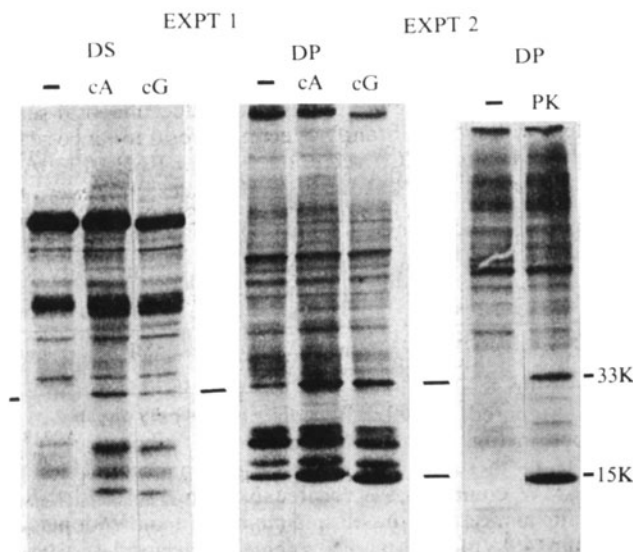


Fig. 3 The effect of cyclic AMP, cyclic GMP and the catalytic subunit of the cyclic AMP-dependent protein kinase on phosphorylation of membrane and cytoplasmic proteins. Membrane vesicles (DP) and cytoplasmic fraction (DS) were prepared as described in Fig. 3. Before phosphorylation the membrane vesicles shown in experiment 2 were lysed in 10 mM HEPES pH 7.3, pelleted and resuspended. This procedure removes from the membrane residual cytoplasmic proteins and loosely associated peripheral proteins. Samples were incubated (after a 2 min incubation at 37°C) for 1 min (expt 1) or 1.5 min (expt 2) at 37°C in 10 mM (expt 1) or 5 mM (expt 2) MgCl_2 , 10 μM [γ - ^{32}P]ATP and 10 mM HEPES pH 7.3. The phosphorylation reaction was stopped by adding an equal volume of gel buffer (4% w/v SDS, 20% glycerol, 4% (v/v) β -mercaptoethanol, 118 mM Tris- PO_4 pH 6.9, 0.008% bromophenol blue). The samples were heated at 98°C for 2 min then frozen before analysis by SDS gel electrophoresis. Autoradiograms of SDS gels of phosphorylated DP and DS are shown. Control channels are indicated (-). Additions present during phosphorylation were 10^{-6} M cyclic AMP (cA), 10^{-3} M cyclic GMP (cG), 30 nanomolar units per ml of catalytic subunit of the cyclic AMP-dependent protein kinase (PK). [Where 1 nmol unit is the amount of enzyme which catalyses the transfer of 1 nmol of ^{32}P label from ATP to 1 mg of protamine sulphate per min.] The catalytic subunit of the cyclic AMP-dependent protein kinase was prepared from bovine lung according to the method of Rosen and Erlichman²³ and assayed according to the method of Walsh *et al.*²⁴.

ribonuclease reduces metabolically incorporated, trichloroacetic acid-precipitable ^3H -uridine by 96%.

The 33,000-MW protein can be phosphorylated *in vitro* by endogenous kinases in isolated cell fractions (DP and DS). The phosphorylation of this component is dependent on the addition of either cyclic AMP or cyclic GMP (Fig. 3). Higher concentrations of cyclic GMP (10^{-3} M) than cyclic AMP (10^{-6} M) are required for maximal phosphorylation of this 33,000-MW protein. The 33,000-MW protein, phosphorylated in the presence of cyclic AMP *in vitro* (Fig. 3), comigrates on SDS gel electrophoresis with the 33,000-MW protein, the phosphorylation of which is increased after addition of FGF to ^{32}P -labelled cells. The 33,000-MW protein is also phosphorylated when the membranes are incubated with a purified cyclic AMP-dependent protein kinase or its catalytic subunit (Fig. 3). The phosphorylation of a protein with an apparent MW of 15,000 is also stimulated *in vitro* by cyclic nucleotides and is increased by the cyclic AMP-dependent protein kinase or its catalytic subunit (Fig. 3). The phosphorylation of this protein is low in ^{32}P -labelled cells and is not increased after the addition of FGF or serum to intact cells (Figs 1 and 2). Because the addition of FGF to intact, ^{32}P -labelled 3T3 cells results in a specific increase in phosphorylation of the 33,000-MW protein mainly in the fraction enriched in plasma membrane, and phosphorylation of both the 33,000-MW protein and a 15,000-MW protein can be increased in all fractions made accessible to cyclic AMP or protein kinase by cell breakage, it appears that, in the intact cell, local regulation of phosphorylation in response to FGF occurs at the membrane.

In summary, the addition of serum or a highly-purified fibroblast growth factor to intact, quiescent 3T3 cells causes a

rapid increase in phosphorylation of membrane proteins, with the most dramatic change in phosphorylation of a protein of MW 33,000. Studies with broken-cell preparations show that the phosphorylation of the 33,000-MW protein is stimulated by cyclic nucleotides, particularly by cyclic AMP.

Possible mechanisms for the increased phosphorylation of the 33,000-MW protein are: (1) an increase in the local concentration of cyclic GMP^{12,13} or cyclic AMP¹⁴; (2) a direct stimulation of a membrane-associated protein kinase by the mitogen, as demonstrated by Carpenter *et al.*¹⁵; they find that epidermal growth factor increases phosphorylation of membrane preparations from human epidermoid carcinoma A-431 cells; (3) translocation of a protein kinase from one cellular compartment to another¹⁶⁻¹⁸; (4) inhibition of a specific phosphatase; or (5) exposure of phosphorylatable sites on the 33,000-MW protein substrate as a result of rearrangement of the protein in the membrane.

The rapidity of the increase in phosphorylation of the 33,000-MW protein after addition of mitogens raises the possibility that phosphorylation of specific membrane proteins may be the initial metabolic event which occurs when a mitogen stimulates quiescent 3T3 cells to synthesise DNA.

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Role of Ca^{2+} -dependent regulator protein in intestinal secretion

AFTER exposure to secretagogues the small intestine changes from a tissue that absorbs fluid and electrolyte from lumen to blood into a tissue that secretes electrolyte and fluid into the lumen¹⁻⁴. It has been shown that this secretion results from an increase in the passive Cl^- permeability of the mucosal border, which permits NaCl to leak passively from the lateral intercellular spaces, where it is present at hypertonic concentrations⁵, into the mucosal bathing solution. Na^+ and water, electro-

Table 1 Fluxes across sheets of rabbit ileum stripped of serosa and external muscle layers

		$J_{\text{m}}^{\text{Cl}^-}$ ($\mu\text{mol cm}^{-2} \text{h}^{-1}$)				
		Control	Cholera-gen ($2 \mu\text{g ml}^{-1}$)	Theophylline (10 mM)	A23187 ($2 \mu\text{g ml}^{-1}$)	A23187 ($2 \mu\text{g ml}^{-1}$) + Theophylline (10 mM)
No addition		11.93	8.64*	7.03†	7.12†	7.86†
		± 0.60	± 0.60	± 0.34	± 0.37	± 0.34
		(45)	(11)	(14)	(26)	(12)
Stelazine (0.1 mM)		11.27	13.13‡	11.14‡	13.88‡	12.00‡
		± 1.26	± 0.20	± 1.15	± 0.60	± 0.82
		(6)	(7)	(4)	(5)	(7)
RMI 12330A (0.1 mM)		11.96	11.45‡	11.65‡	12.22‡	11.02‡
		± 0.43	± 0.54	± 0.84	± 0.40	± 0.45
		(17)	(9)	(6)	(25)	(5)

The exposed area was 1.76 cm^2 (ref. 10). Bathing solutions contained in addition to secretagogues, mM: 140 NaCl, KHCO_3 , 2.4 K_2HPO_4 , 0.4 KH_2PO_4 , 1.2 CaCl₂ and 1.2 MgCl₂. Solutions were maintained at 37 °C and gassed with 5% CO₂; 95% O₂. ^{36}Cl was added to mucosal solution, to measure m-s flux, or serosal solution for s-m flux; the cold sides were sampled at 30-min intervals for 1.5 h. All secretagogues were present at equal concentrations on both sides of the tissue. The cholera-gen was supplied by Schwarz-Mann. No significant effects of treatment on $J_{\text{m}}^{\text{Cl}^-}$ were observed. The pooled mean of fluxes measured in all conditions gave $J_{\text{m}}^{\text{Cl}^-} = 7.88 \pm 0.16(60) \mu\text{mol cm}^{-2} \text{h}^{-2}$. Values are mean $\pm$ s.e.m.

* $P < 0.005$; test compared with control (column 1).

† $P < 0.001$; test compared with control.

‡ $P < 0.001$; test compared with treated without modifier.

osmotically coupled to Na⁺ movement, leak through the tight junctions^{1,2}, and Cl⁻ leaks through relatively anhydrous anion-selective channels, induced within the mucosal border by secretagogues. The increased reflux of NaCl from the lateral intercellular space accounts for both the apparent decrease in electroneutral NaCl uptake across the mucosal border induced by secretagogues and the apparent increase in active Cl⁻ secretion and short-circuit current^{3,6,7}. We have investigated the mechanism by which intestinal secretagogues increase passive Cl⁻ permeability and thereby cause secretion. Cl⁻ permeability is increased by several secretagogues, some of which, such as theophylline and cholera-gen, increase intracellular cyclic AMP concentration, and others, such as A23187, the Ca²⁺ ionophore, or carbachol, do not⁸. Thus there has been no known common mode of secretory induction. To investigate this problem we used two drugs that prevent intestinal secretion *in vitro*, RMI 12330A (Richardson Merrell), and the antipsychotic phenothiazine trifluoperazine (Stelazine, Smith, Kline and French). RMI 12330A prevents secretion by inhibiting cholera-gen-induced adenylyl cyclase activity⁹. Stelazine inhibits phosphodiesterase in tissues^{11,12} by preventing the activation of the

Table 2 Passive Cl⁻ flux across sheets of rabbit ileum with serosa and muscle layers removed

		$J_{\text{m}}^{\text{Cl}^-}$ ($\mu\text{mol cm}^{-2} \text{h}^{-1}$) $\pm$ s.e.m.	
		Control	Theophylline (10 mM)
No addition		10.65 $\pm$ (0.34) (56)	14.00* $\pm$ 0.55 (32)
Stelazine (0.1 mM)		11.65 $\pm$ 0.78 (3)	11.81† $\pm$ 0.47 (8)
RMI 12330A (0.1 mM)		10.65 $\pm$ 0.41 (30)	10.20† $\pm$ 0.60 (24)

The exposed area was 1.76 cm^2 . The mucosal bathing solution contained mM: 200 NaCl, 10 KHCO_3 , 2.4 K_2HPO_4 , 0.4 KH_2PO_4 , 1.2 CaCl₂ and 1.2 MgCl₂. The serosal solution contained: 280 mannitol, 10 KHCO_3 , 2.4 K_2HPO_4 , 0.4 KH_2PO_4 , 1.2 CaCl₂ and 1.2 MgCl₂. Both mucosal and serosal solutions contained 0.1 mM ouabain to inhibit active transport. The solutions were maintained at 37 °C and gassed with 5% CO₂; 95% O₂. ^{36}Cl was added to the mucosal solution and the serosal side was sampled at 30-min intervals for 1.5 h. All other reagents were present at equal concentrations in both bathing solutions.

* $P < 0.001$; test compared with control.

† $P < 0.001$; test compared with theophylline.

enzyme by Ca²⁺-dependent regulator protein, CDR. We report here that it also inhibits Cl⁻ secretion and binds to CDR.

Table 1 shows that theophylline (10 mM), cholera-gen ($1 \mu\text{g ml}^{-1}$) and A23187 ($2 \mu\text{g ml}^{-1}$) all reduce mucosal-serosal Cl⁻ flux, without significantly affecting serosal to mucosal flux, thereby inducing net Cl⁻ secretion. Neither RMI 12330A nor Stelazine affect Cl⁻ flux in control tissues; however, both prevent the decreases in mucosal-serosal Cl⁻ fluxes induced by the secretagogues. Table 2 shows that Stelazine and RMI 12330A prevent the increase in passive Cl⁻ permeability across the mucosal border induced by theophylline.

Table 3 Effect of RMI 12330A and Stelazine on cyclic AMP after exposure to secretagogues

		cyclic AMP (pmol per mg tissue dry weight)				
		Control	Cholera-gen ($2 \mu\text{g ml}^{-1}$)	Theophylline (10 mM)	A23187 ($2 \mu\text{g ml}^{-1}$)	A23187 ($2 \mu\text{g ml}^{-1}$) + Theophylline (10 mM)
No addition		9.50	19.00*	44.60*	9.67	70.00*
		± 0.57	± 0.88	± 2.95	± 0.33	± 5.35
		(17)	(7)	(9)	(19)	(8)
Stelazine (0.1 mM)		9.75	20.26*	51.21*	13.00*‡	67.44*
		± 0.34	± 1.24	± 5.10	± 0.27	± 6.60
		(6)	(4)	(9)	(13)	(3)
RMI 12330A (0.1 mM)		7.70	10.48†	9.30†	7.80†	41.36*†
		± 0.84	± 0.94	± 0.61	± 0.30	± 3.20
		(12)	(6)	(12)	(5)	(4)

Tissues were incubated for 30 min before extraction, except where exposed to cholera-gen with or without modifiers, when they were incubated for 60 min. Approximately 0.1-g pieces of tissue were homogenised in a glass homogeniser with a Teflon pestle in 2 ml of ice-cold 4 mM EDTA, pH 7.4. The homogenates were then boiled for 2 min and the cyclic AMP of aqueous extract was measured using a cyclic assay kit (Radiochemical Center, Amersham). Values are mean $\pm$ s.e.m.

* $P < 0.001$; test compared with control (column 1).

† $P < 0.001$; test compared with treated without modifier (row 1).

‡ $P < 0.005$; test compared with treated without modifier.

Table 3 shows the effect of RMI 12330A and Stelazine on the induced changes in intracellular cyclic AMP after exposure to various secretagogues. Theophylline and cholera-gen increased tissue levels of cyclic AMP; A23187 had no effect on them. With theophylline present, A23187 increased cyclic AMP levels above those found with theophylline alone, suggesting that raised intracellular Ca²⁺ increases both adenylyl cyclase and phosphodiesterase activity. RMI 12330A prevented the increase in tissue levels of cyclic AMP seen with theophylline and cholera-gen. This effect is consistent with its reported effects as an inhibitor of adenylyl cyclase activity⁹. Stelazine had no effect on tissue levels of cyclic AMP in controls, or in conditions where they were already high due to the presence of theophylline or cholera-gen.

These results indicate that Cl⁻ secretion is independent of tissue levels of cyclic AMP because secretion was observed either with high levels of cyclic AMP, in the presence of theophylline or cholera-gen, or with low levels of cyclic AMP, in the presence of A23187. And this secretion was in all conditions inhibited by Stelazine, which did not affect intracellular cyclic AMP levels. However, our results do indicate that adenylyl cyclase is activated whenever secretion is observed.

A single event leading to activation of secretion is probable. A likely common event in all stimulating modes is a rise in free Ca²⁺ within the tissue. With A23187 this can be assumed from its well known action in promoting increased membrane permeability to Ca²⁺ (ref. 10). However, as the small intestine has a very active Ca²⁺ pump, it is hard to demonstrate increased uptake even of labelled Ca²⁺ with this agent, and very hard to show directly any change in intracellular free Ca²⁺ levels. CDR has a raised affinity for Stelazine in the presence of $1 \mu\text{M}$ Ca²⁺ (refs 11, 12). This high affinity for Stelazine is specific to the Ca-CDR complex. CDR alone has only low affinity for Stelazine. We therefore examined the uptake of ³H-labelled

Table 4 Uptake of ^3H -Stelazine

		Stelazine ($\mu\text{mol per g wet weight}$)			
		Control	Cholera-gen ($3\mu\text{g ml}^{-1}$)	Theophylline (10 mM)	A23187 ($2\mu\text{g ml}^{-1}$) + EGTA (0.5 mM)
No addition	1.00	3.31*	2.02*	2.36*	1.06
	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
	0.03 (38)	0.12 (9)	0.11 (20)	0.15 (19)	0.06 (6)
RMI 12330A (0.1 mM)	1.08	0.26†	1.14§	1.10§	0.68†
	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
	0.06 (9)	0.02 (8)	0.05 (3)	0.37 (3)	0.07 (5)

Uptake of ^3H -Stelazine into pieces of tissue of approximately 0.1 g after incubation in Ringer solution for 30 min, except with cholera-gen where incubation was for 50 min. Stelazine required 15 min to reach equilibrium with the tissue. The drug was extracted for 18 h in 2 ml of 0.1 N HNO_3 . The initial concentration of ^3H -Stelazine was $0.3\text{ }\mu\text{M}$. (34.9 mCi mM^{-1}). Values are mean $\pm$ s.e.m.

* $P < 0.001$; test compared with control (column 1).

† $P < 0.001$; test compared with treated without modifier (row 1).

‡ $P < 0.005$; test compared with control.

§ $P < 0.005$; test compared with treated without modifier.

Stelazine into rabbit ileum to see if Ca^{2+} -dependent Stelazine-binding could be used as an indicator of raised free intracellular Ca^{2+} levels in the secretory state.

Table 4 shows that Stelazine uptake by rabbit ileum was stimulated by A23187, cholera-gen and theophylline and inhibited by RMI 12330A. The A23187-stimulated uptake was dependent on the presence of Ca^{2+} in the external Ringer solution. The apparent dissociation constant of Stelazine for intracellular sites in theophylline-stimulated rabbit intestine was 10^{-6} M . This is in close agreement with previous work on the affinity of Stelazine for purified CDR¹¹⁻¹³.

These results are consistent with the view that intracellular free Ca^{2+} is raised to approximately 10^{-6} M during all types of secretory stimuli, as indicated by the increase in high affinity Stelazine-binding within the tissue. Because the stimulation by A23187 of intestinal Cl^- secretion can be blocked by Stelazine or RMI 12330A, it is clear that raised intracellular Ca^{2+} by itself does not stimulate secretion, but that the Ca^{2+} -activated form of CDR may directly activate the increase in mucosal border Cl^- permeability.

To ascertain whether RMI 12330A and Stelazine act on Ca-CDR, we extracted CDR from rabbit small intestine by the method of Dedman *et al.*¹⁴. We found that RMI 12330A competed with ^3H -Stelazine for the Ca^{2+} -dependent binding sites on the protein extract.

We conclude that activation of intestinal secretion involves the following sequence: secretagogues, or ionophore, bind to the brush-border and increase passive Ca^{2+} uptake into the epithelial cells; the increased Ca^{2+} uptake increases intracellular Ca^{2+} activity to approximately 10^{-6} M . At this concentration Ca^{2+} binds to CDR to form Ca-CDR, which then activates the brush-border Cl^- conductance, thus permitting increased NaCl leakage from the lateral intercellular space—hence secretion. Additionally, Ca-CDR may activate adenylyl cyclase and phosphodiesterase, but the steady state concentration of cyclic AMP seems not to be an important parameter in the control of secretion.

Binding of antipsychotic phenothiazines to Ca^{2+} -CDR prevents the activation of several enzyme reactions^{11,15} and here is shown to inhibit the activation of brush-border Cl^- conductance. It has been reported that chlorpromazine, a weak antipsychotic phenothiazine, reduces intestinal fluid loss caused by cholera in human subjects¹⁶. This drug, like trifluoperazine, binds to Ca-CDR, albeit with a lower affinity^{11,17}. Our results suggest that it might be worth investigating a range of phenothiazines for anti-diarrhoeal activity, having regard to their affinities for Ca-CDR, rather than their effectiveness as inhibitors of adenylyl cyclase.

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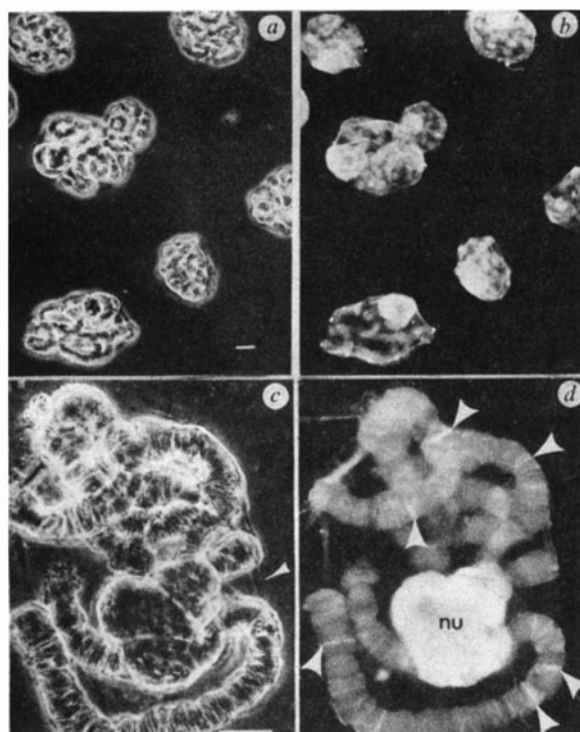
Concanavalin A binds to puffs in polytene chromosomes

CHANGES in transcriptional activity at defined loci are often correlated with significant local structural changes in the genome¹, and in polytene chromosomes, such changes are thought to be associated with compositional or conformational changes in the protein complement at these particular bands^{2,3}. Thus, various studies on Balbiani rings and specific 'puffs' in such chromosomes are useful for elucidating the role of defined chromosomal components in both chromosome structure and gene activity. Such studies require specific probes which will allow *in situ* localisation of a chromosomal component during the various stages of puffing. Antibodies specific to purified histone fractions⁴⁻⁷, HMG proteins⁸, RNA polymerase⁹ and non-histone protein subfractions¹⁰ have been used in studies on chromatin and chromosome structure. We reported previously that concanavalin A (Con A) specifically binds to three types of non-histone proteins present in chromatin purified from rat liver nuclei and suggested that derivatives of Con A might serve as specific probes to study the *in situ* organisation of these non-histone proteins¹¹. We have now reacted fluorescein-labelled Con A with polytene chromosomes isolated from different developmental stages of *Chironomus thummi* and visualised the location of the bound Con A by fluorescence microscopy. We observed that the fluorescent lectin, which has an affinity for glucose- and mannose-containing molecules, specifically bound to the transcriptionally active regions of chromosome IV. The extent of binding of Con A to the Balbiani rings present in regions b and c of chromosome IV is proportional to the size of the respective ring. Our results indicate that glucose- or mannose-containing molecules are present in these Balbiani rings and that the availability of these sugars to interact with Con A can be correlated with the developmental stage of a puff. We suggest that lectins can be useful cytological tools with which to study the *in situ* organisation of defined chromosomal components during various functional states of the genome.

To avoid background staining due to Con A binding sites present in plasma membranes¹², it was necessary to isolate unfixed nuclei containing polytene chromosomes from *Chironomus* salivary glands. As shown in Fig. 1a, these nuclei are completely free of cytoplasmic material. When such nuclear

preparations are reacted with fluorescein-labelled Con A and examined using fluorescence microscopy, the outline of the chromosomes is clearly visible (Fig. 1b). Closer examination of the fluorescence pattern reveals banding of the Con A in the chromosomes (Fig. 1d), and preferential binding to distinct regions can be detected (arrows). The intensity of fluorescence is proportional to the concentration of fluorescein-labelled Con A used. The binding is specific, because (1) it is abolished when the nuclei are preincubated with unlabelled Con A; (2) it is abolished when the nuclei are preincubated with carbohydrates for which Con A has a specific binding site, such as α -methyl-D-mannoside and glucose; (3) it is not abolished when the nuclei are incubated with galactose, a sugar with a low affinity for Con A. Thus, the ability of various sugars to inhibit the binding of fluorescent Con A to polytene chromosomes parallels its specificity for various sugars^{12,13} and agrees with our studies done in purified chromatin isolated from rat liver¹¹.

To study the possible correlation between transcriptional activity and the presence of Con A binding sites in polytene chromosomes, the binding of Con A to Balbiani rings IVb and IVc of *C. thummi* was investigated. The size and transcriptional activity of these two puffs are correlated with the developmental state of the larvae¹⁴. Nuclei were purified from salivary glands obtained from larvae at various developmental stages, and chromosome IV with its attached nucleolus isolated¹⁵. To prevent protein migration or rearrangement the isolated



chromosomes of *C. thummi* salivary gland nuclei. Nuclei were isolated¹⁵, transferred through 4 drops of *Chironomus* Ringer's, pH 6.3 (ref. 15) and mounted in a small drop of fixer solution (87 mM NaCl, 2 mM MgCl₂, 10 mM phosphate buffer, pH 7.3, 5% formaldehyde) using a siliconised coverslip and an untreated slide. The coverslip was floated off and the preparation fixed for 5 min in fixer solution, washed for 1 h in NCMT-buffer (87 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, 0.2% Triton X-100) and then transferred to NCMT-buffer without Triton X-100. Fifty μ l of fluorescein-labelled Con A (Miles) diluted 1:400 were applied to the preparation and these were incubated for 1 h at 4°C in a moist chamber; the preparation was then washed for 18 h. a, Phase contrast micrograph; b, corresponding fluorescence micrograph; c, phase contrast micrograph of nucleus at higher magnification. Arrow indicates nuclear membrane; d, corresponding fluorescence micrograph. Arrows indicate intensely fluorescent chromosomal regions. nu designates the nucleolus. Scale bar, 10 μ m.

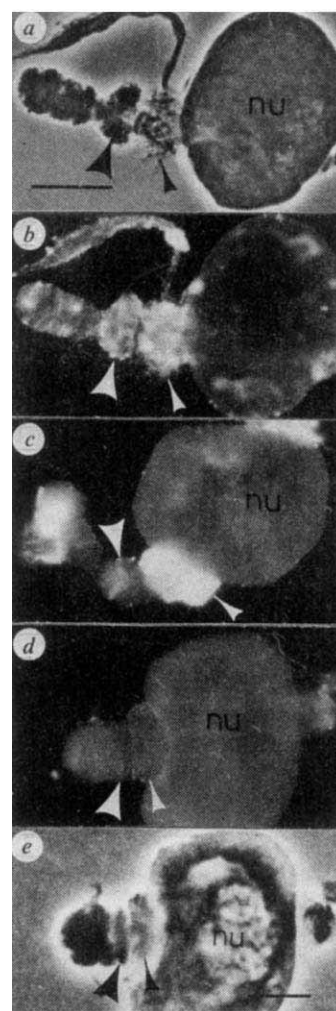


Fig. 2 Chromosome IV at different developmental stages reacted with fluorescein-labelled Con A. Chromosome IV was isolated from unfixed purified nuclei and then treated as in Fig. 1 except that the fluorescein-labelled Con A was diluted 1:800. a, Phase contrast micrograph of chromosome IV at late larval stage; b, corresponding fluorescence micrograph; c, fluorescence micrograph of chromosome IV at late prepupal stage; d, fluorescence micrograph of chromosome IV at red pupa stage; e, corresponding phase contrast micrograph. Large arrows denote the Balbiani ring in region b. Small arrows designate the Balbiani ring in region c. Chromosome regions according to Keyl¹⁶. Developmental stages according to Laufer¹⁴. Scale bar, 10 μ m.

chromosomes were fixed with formaldehyde. The formaldehyde-fixed chromosomes were treated with either fluorescein- or rhodamine-labelled Con A and examined using phase and fluorescence microscopy.

The results presented in Fig. 2 reveal a correlation between the size of the puff and its ability to bind Con A. Thus, the two puffs clearly visible in the phase contrast micrograph in Fig. 2a display strong fluorescence (Fig. 2b). Note that the larger, less condensed puff IVc (indicated by small arrow) fluoresces more intensely than the more condensed puff IVb (indicated by large arrow). At a later stage of development, puff IVb becomes condensed while puff IVc remains decondensed. As shown in Fig. 2c, puff IVc retains its ability to bind Con A. Figure 2e shows a phase micrograph of chromosome IV typical of the red pupa stage. Both puffs IVb and IVc are condensed. The corresponding fluorescence micrograph (Fig. 2d) reveals that these regions lost their ability to bind Con A. Autoradiographic studies with ³H-Con A verified these observations. Thus, the autoradiograph of a chromosome typical of the late pupa stage, in which puff IVc is larger than puff IVb, displays stronger labelling in region IVc.

The changes in the ability of the Balbiani rings to bind Con A may reflect either migration of particular sugar-containing components into the puffs or rearrangement of these components during the various stages of puffing.

Considering the possibility that the saliva of the gland contains Con A-binding components which might bind to the chromosomes during the isolation procedure, we note that: (1) the isolated nuclei were extensively washed and in the intact nuclei Con A binding is present along the chromosome axis and absent from the nucleoplasm; (2) the Con A-binding compounds seem to be preferentially concentrated in specific chromosomal regions during certain developmental stages. Thus, even if the presence of the Con A-binding component represents a secondary binding phenomenon, it is still specific and can potentially serve as a marker for structural changes occurring at defined loci in the chromosome.

The nature of the biochemical entity to which Con A binds is unknown; however, it has been reported that puffing is associated with an accumulation of non-histone proteins^{2,3} and we have detected glycoproteins among the non-histone chromosomal proteins¹¹.

Changes in the non-histone protein complement associated with puffing have been observed using serological techniques¹⁰. We have noted that experimentally induced puffs in both *Drosophila* and *Chironomus* as well as the two Balbiani rings studied here do not stain at all or stain very weakly with antisera to specific histone fractions^{4,17}. The present report demonstrates that in a manner analogous to antibodies it is possible to use Con A as a cytological probe to detect and visualise structural changes associated functional changes occurring in the genome.

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Detection of singlet oxygen production by ESR

SINGLET molecular oxygen is a very powerful oxidant. Its action is important in a variety of chemical and biological processes¹⁻⁴, for examples dye-sensitised photooxidation of lipids, proteins and nucleic acids⁴, photodynamic inactivation of viruses⁵ and cells⁴, phototherapy of cancer^{6,7}, carcinogenesis⁸, haemolysis of erythrocytes⁹, sensitisation of the human skin⁴ and

Table 1 ESR parameters for the photo-produced radical and for TAN

	g-factor	Linewidth (G)	Splitting from N (G)
Photo-produced radical	$2.0068 \pm 3 \times 10^{-4}$	0.41 ± 0.02	16.08 ± 0.10
TAN	$2.0068 \pm 3 \times 10^{-4}$	0.39 ± 0.02	16.10 ± 0.10

degradation of food⁴. The methods used to detect singlet oxygen are unspecific, of low sensitivity or laborious. Photooxidation of 1,3-diphenylisobenzofuran seems to be the most widely used diagnostic test for ¹O₂. However, in the absence of additional control experiments this test does not prove the intermediacy of ¹O₂ (ref. 4) and 1,3-diphenylisobenzofuran has very low solubility and dimerises in aqueous solutions. Lion *et al.*¹⁰ have proposed a new method to detect ¹O₂ involving the generation of stable nitroxide radicals when ¹O₂ reacts with the sterically hindered amine 2,2,6,6-tetramethylpiperidin. When using this method to detect ¹O₂ in neutral aqueous solutions, we found no radical production. We report here our investigation of this problem, as it is biologically important to be able to detect ¹O₂ production in such solutions.

Aqueous solutions of four heterocyclic amines were investigated. ¹O₂ was produced photochemically by irradiating the solutions in the presence of 10⁻⁴ M haematoporphyrin. Haematoporphyrin is an efficient photosensitiser which acts mainly through the singlet oxygen mechanism^{11,12}. A correspondence between the titration curves and the radical yields was found (Fig. 1). The molecules of the heterocyclic amines used in this work are neutral on the alkaline side of the titration curve and protonated at the nitrogen atom on the acid side. Figure 1 shows that only neutral molecules react with ¹O₂ to produce nitroxyl radicals, and also indicates that the heterocyclic amines must have pK values lower than about 8 to be suitable for ¹O₂ detection in neutral solutions. Only one of the four amines tested in this work fulfils this requirement: 2,2,6,6-tetramethyl-4-piperidone, which has a pK value of 7.6. The product of the reaction of ¹O₂ with this amine is expected to be 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TAN). This was verified by comparing the ESR spectrum of the photo-produced

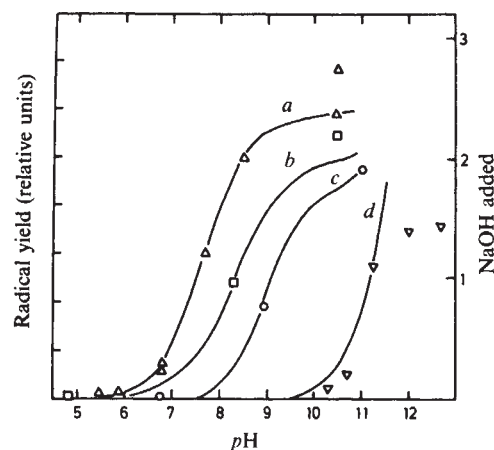


Fig. 1 Titration curves (lines) and yields of photo-produced radicals in aqueous solutions of sterically hindered amines. The amines are: a, Δ : 0.02 M 2,2,6,6-tetramethyl-4-piperidone, b, $\square$: 0.02 M 2,2,5,5-tetramethyl-3-pyrroline-2-carboxamide, c, $\circ$: 0.02 M 2,2,5,5-tetramethyl-pyrroline-3-carboxamide, and d, ∇ : 2,2,6,6-tetramethyl-piperidin. The samples contained 10⁻⁴ M haematoporphyrin as a photosensitiser and were irradiated with a 200 W Osram high-pressure mercury lamp fitted to a Bausch & Lomb grating monochromator ($\lambda = 405$ nm). Irradiation and registration took place in the cavity of an X-band ESR spectrometer of the reflection type. The radical yields were monitored by measuring the slopes of the radical concentration-time curves and adjusted to fall on the titration curves at the pK values. Temperature 22 °C.

radicals with that of TAN, which was synthesised according to the method described by Rozantsev¹³. As seen from Table 1 the linewidth, the *g*-factor and the nitrogen splitting of the photo-produced radical and those of TAN are identical within the error limits.

The photo-induced yield of radicals was about 10 times higher in D₂O than in H₂O. This confirms our assumption that the radicals are produced by a singlet oxygen pathway as the lifetime of ¹O₂ is 10 times longer in D₂O than in H₂O (ref. 14). Furthermore, this shows that 0.02 M 2,2,6,6-tetramethyl-4-piperidone does not reduce the lifetime of ¹O₂ even in D₂O. Thus the method is convenient for studying the effect of singlet oxygen quenchers.

When using this method to monitor singlet oxygen yields in different aqueous solutions pH as well as the temperature of the solutions should be carefully controlled. The temperature dependence of the yields follows the Arrhenius equation between 5 and 40 °C. An activation energy of 12.5 kcal mol⁻¹ may be estimated for the reaction of singlet oxygen with 2,2,6,6-tetramethyl-4-piperidone (0.3 M at pH = 7.4). Furthermore, one should be aware that any strong oxidising agent may produce the radical: for example when TAN is synthesised from 2,2,6,6-tetramethyl-4-piperidone, H₂O₂ is used as the oxidising agent¹³. Hence, in systems where many oxidants are likely to be produced one should compare the yields with H₂O and D₂O as solvents to verify that radical production takes place through singlet oxygen.

The sensitivity of the method was estimated as follows: Using a known concentration of TAN as a standard and determining the light intensity ($\lambda = 405$ nm) by means of a calibrated YSI Kettering Model 65A radiometer we were able to estimate the quantum yield of photo-produced radicals to 5×10^{-4} in a solution of 0.3 M 2,2,6,6-tetramethyl-4-piperidone and 10^{-4} M haematoporphyrin (pH = 7.4). Before irradiation about 4×10^{-7} M radicals were present in the samples as an impurity. To produce an additional 4×10^{-7} M radicals in an ESR sample of 0.2 ml a light dose of 50 mJ must be absorbed in the sample. The sensitivity of our ESR spectrometer allows detection of 10^{-8} M TAN so one could, in principle, detect an absorbed dose of 1.3 mJ provided one could get rid of the background signal by purifying the amine.

These doses may be compared with biologically relevant doses. The D₃₇ dose for inactivation of NHK 3025 cells, which originate from a human carcinoma *in situ*, is about 1 J cm⁻². This refers to cells growing in tissue culture flasks being irradiated ($\lambda = 405$ nm) in the presence of 4×10^{-4} M haematoporphyrin in E2a medium (J.M., unpublished results). Light doses proposed for phototherapy of cancer are of the order of 120 J cm⁻² (ref. 15). Thus in such conditions the method of detecting singlet oxygen by means of its reaction with 2,2,6,6-tetramethyl-4-piperidone has a sensitivity which is more than adequate.

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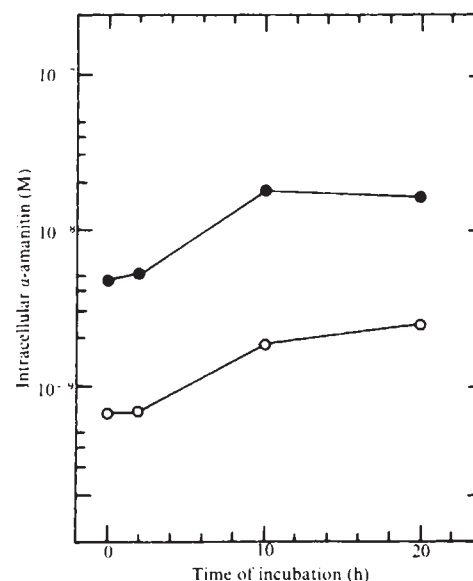
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Corrigenda

In the letter 'Viroid replication is inhibited by α -amanitin' by E. W. Ferguson and G. E. Sanger, *Nature* **278**, 185, the values for α -amanitin concentration on Fig. 2 are incorrect. The figure is reproduced correctly below.



In the article 'Bursal dissections and gill pouch hormones' by J. W. Shields, *Nature* **259**, 373-376 (1976), the following items on page 374 (column 2) should be amended:

(1) In line 1, for surgical bursectomy read testosterone bursectomy.

(2) In section (4) it was stated that birds also differ from mammals because avian neonates lack immunoglobulins from maternal milk. This is true in precocial hatchlings (such as chicks), but is not true for altricial nestlings (such as pigeons) which feed until nidifugation on regurgitated parental crop sac milk¹. The 'pigeon's milk' is produced in parental pharyngeal mucous glands containing tonsillar tissue; it looks like mammalian colostrum; and apparently compensates for the atrophic cloacal bursae of *Fabricius* in altricial nestlings².

1. Welty, J. C. *The Life of Birds* (Saunders, Philadelphia, 1975).

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Erratum

In the letter 'Highly efficient quantum conversion at chlorophyll *a*-lecithin mixed monolayer coated electrodes' by Miyasaka *et al.*, *Nature* **277**, 638-640, Table 1 (shown below) was omitted.

Table 1 Photocurrent quantum efficiencies at chlorophyll *a*-lecithin mixed monolayers*

Molar ratio Chl <i>a</i> /DPL	Mean Chl <i>a</i> -Chl <i>a</i> intermolecular distance (Å)	Absorbance per layer† in red band	Peak positions of photocurrents (nm)		Photocurrent efficiency in red band‡ (%)
			Red	Blue	
1/0	10	0.0082	675	415	6
2/1	12	0.0060	675	415	8
1/1	12	0.0058	675	412	10
1/2	13	0.0047	675	415	9
1/4	17	0.0031	675	415	8
1/9	24	0.0018	673	418	10
1/19	36	0.0008	673	410-420	14 ± 2
1/49	56	0.0003	666	420	25 ± 5
1/99	83	0.0002	670	425	25 ± 5

* Conditions of measurements: electrolyte composition, 0.05M H₂O, 0.1M Na₂SO₄ and phosphate buffer (pH 6.9); electrode potential, 0.1V vs SCE; surface pressure for monolayer deposition, 20 dyn cm⁻¹.

† Measured at OTE-air interface.

‡ In the absence of H₂O, the quantum efficiency measured at 0.6V vs SCE in neutral electrolyte solution was about 1.5% for a mixed monolayer of Chl *a*/DPL (1/1).

matters arising

Nautiloid growth rhythms and lunar dynamics

We have received several interesting comments on the controversial paper by Kahn and Pompea and have decided to publish only a representative selection. With the contributions below we, therefore, close the discussions on nautiloid growth rhythms.

KAHN AND POMPEA¹ claim to have identified daily growth lines and lunar monthly septa in *Nautilus pompilius* shells of various ages back to the Upper Ordovician. They thus count the number of days in the month (obtaining in Recent shells the present value of 29.5) and find a monotonically decreasing number as older geological periods are sampled until for the Upper Ordovician a mean value of 9 is found. They claim that these data prove that the Moon's average rate of recession from the Earth since the Ordovician due to lunar tidal friction was 17 times as great as the value (5.8 cm yr^{-1}) in the past 2,000 yr. Their curve of growth lines per chamber plotted with time shows variations in the lunar rate of recession by a factor of 3 only.

Their calculations, however, are fundamentally in error. They use Kepler's third law alone to determine the semi-major axis of the lunar orbit for each geological period. To obtain the length of the month they multiply their counts by the length of the day in the geological past, obtaining this by simply assuming that it has increased by 2.5 ms per century. The latter value is obtained from astronomical data and from previous palaeontological studies². The synodical month, to which marine life would respond, must, of course, be converted to the sidereal month for use in Kepler's equation and this correction they make. Otherwise, their procedure is incorrect as the rate of retreat of the Moon of 5.8 cm yr^{-1} is consistent with a lengthening of the day of 2.5 ms per century while their claimed value of 94.5 cm yr^{-1} is inconsistent.

Their calculation should have used both Kepler's second and third laws. Neglecting the ellipticity of the Moon's orbit, I have shown³ that $G^2 T \propto L^3$, and $Gr \propto L^2$ for any geological time where G is the universal gravitational constant, T the length of the month, r the Earth-Moon

distance and L the orbital angular momentum of the Moon. A further equation connecting the loss of spin angular momentum of the Earth to the gain of orbital angular momentum of the Moon must be used. If t and t_0 are the past and present lengths of the day respectively, then

$$2\pi I/t - 2\pi I/t_0 = (1 + \beta)(L_0 - L)$$

where I and I_0 are the past and present moments of inertia of the Earth and β is the ratio of the solar to the lunar tidal frictional torque, at present $1/5.1$ or $1/3.4$ for linear and nonlinear theories⁴ respectively. If s is the number of Kahn and Pompea's counts of growth lines per septa, then $T/t = s(1 - T/Y)$ where Y is the length of the year. From these three equations, taking β to be constant:

$$\begin{aligned} (s/27.3)[1 - (G/G_0)^{3/2}(r/r_0)^{3/2}/13.4] \\ = (r/r_0)^{3/2}[1 + 4.82(1 + \beta)(1 - (G/G_0)^{1/2} \\ \times (r/r_0)^{1/2})](I_0/I)(G_0/G)^{1/2} \end{aligned}$$

The law of retreat of the Moon from the Earth can then be calculated correctly, assuming that the Earth's moment of inertia has not changed and that G is constant, on the assumption that the counts listed in Kahn and Pompea's Table 1 are really numbers of days in the synodical month. These data are shown in Fig. 1 and may be compared with their incorrect values: their data really implies that the Moon was further away in the past

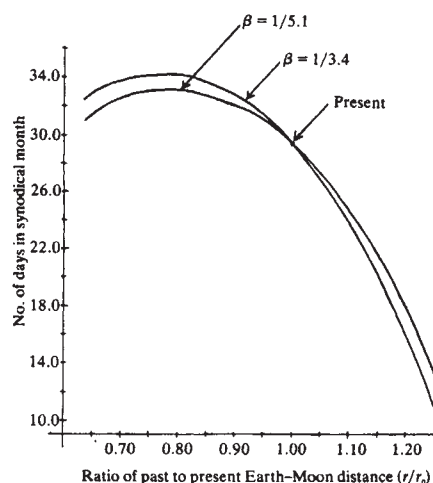


Fig. 1 Rate of retreat of the Moon from the Earth.

than at present. Their claim is not that the Earth-Moon distance was about 0.4 of the present in the Upper Ordovician but that it was 25% greater!

The point is that although by Kepler's third law the month was shorter in the past if lunar tidal friction had caused the Moon to retreat from the Earth, Newton's third law of motion results in the day having been proportionately even shorter: thus the number of days in the month were greater and not smaller in the remote past. The error is to have used a day length too great which results in too few days in the month.

The corrected Kahn and Pompea observations could only be true if the lunar-solar attraction on the Earth resulted in a very large accelerating rather than a decelerating torque! The above equation shows that lunar tidal friction could still have been working through geological time at roughly its present rate if sI/I_0 were always a little greater than 29.5. Thus if I were equal to $3I_0$ in the Upper Ordovician, all would be well, and those⁵, for example, who are reviving the old theory of mountain building by contraction of the Earth would have evidence of a secular diminution in the Earth's moment of inertia but some 100–1,000 times what they need. Similarly the *Nautilus* observations would be consistent with tidal friction working at the present rate if $s(G/G_0)^{1/2}$ was always a little greater than 29.5. A secular decrease in G has been postulated in Dirac⁶ and others but a 70% decrease since the Ordovician is excluded on palaeoclimatic data alone, quite apart from physical objections.

The transfer of angular momentum between the Earth's rotation and the Moon's orbit by the agency of lunar tidal interactions must involve the diminution of the tidal kinetic energy of the system which reappears as heat. It is, of course, easy to calculate from Kahn and Pompea's claims what is implied concerning the amount of heat which must be dumped in the Earth-Moon system since the Ordovician. It is an order of magnitude greater than the heat which has come out of the Earth over this time. If a small fraction were due to dissipation in the Earth's mantle and crust, the thermal history of the Earth would need rediscussion.

I thank Dr G. Turner for helpful discussion.

Note added in proof: Kahn and Pompea

have refused my solution and place the Moon even closer to the Earth (0.2 of its present distance instead of 0.4) in the Ordovician. But as their data shows no rise to the maximum of about 33 d in the synodical month with increasing geological age, their claim is now that in mid-Tertiary the Moon was 0.5 of its present distance and has since retreated at a mean rate 150 times its present rate.

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KAHN AND POMPEA REPLY—In our article detailing the value of nautiloids as potential geochronometers we mentioned that calculations incorporating the nautiloid data can establish the changing length of the day through geological time and that these calculations were in progress¹. Given this independent means of establishing palaeo day lengths, it is unnecessary to rely on coral and eclipse data as these yield only a first order calculation of palaeo Earth–Moon distances. Runcorn has presented equations for a more detailed calculation. The direct implication with Runcorn's calculation is that our nautiloid growth data suggest a Moon further from the Earth in the past. Not only is this unacceptable on geophysical grounds², since the mechanism of tidal friction can presently act only in the sense of increasing the Moon's distance with time, but our solution to the equation yields a Moon much closer in the past. The results we present here are consistent with our original conclusion that tidal frictional forces have varied considerably and were of a much greater magnitude in the geological past than other previously published estimates indicate.

Runcorn's graph is incomplete because it does not include another set of possible solutions to the equations cited. These

solutions reflect a smaller synodic month in conjunction with smaller values of r/r_0 ; that is a closer Moon for lunar months of shorter duration than the present 29.5 d. This can be shown by a careful examination of the roots of the quadratic equation in $(r/r_0)^{1/2}$ which Runcorn cites. Substituting $x = (r/r_0)^{1/2}$ and setting $I_0/I = (G_0/G)^{1/2} = 1$ (the assumptions made by Runcorn), we get

$$x^4 - \{1 + 1/[4.82(1 + \beta)]\}x^3 \\ - \{s/[1763.25(1 + \beta)]\}x \\ + s/[131.59(1 + \beta)] = 0$$

Unfortunately Runcorn did not present the solutions that arise when solving for r/r_0 , given β and s . Instead he apparently presented only the solutions determined by solving for s , given β and r/r_0 . Thus a whole set of solutions was omitted. Table 1 shows both sets of solutions. Those with subscript two are the values plotted in Runcorn's graph.

Either root is a valid solution to the equation—one column does not have to be accepted to the exclusion of the other. Thus, a graph of days per month as a function of increasing years ago rises very gradually above 30, then drops abruptly towards zero³. Most of the nautiloid data would appear on the portion of the curve that drops abruptly. It is seen that the results reported by us previously¹, namely a smaller Earth–Moon distance, are consistent with the general trend of the set of solutions ignored by Runcorn.

Quite apart from these calculations it is evident just from Kepler's third law that a lesser number of hours per sidereal month implies a smaller semi-major axis and a closer lunar orbit. That there may be more days (of shorter length) per month for part of the evolutionary history does not alter the crux of our original calculations.

In conclusion, our calculations which indicate a much closer Moon in Ordovician times are hardly reconcilable with derivations based solely on the magnitude of present tidal friction. If the nautiloid data reflect the orbital parameters of the Earth–Moon system, then additional tidal energy sinks of large magnitude must have been present in past geological periods. These possibilities will be evaluated in a forthcoming paper. Although Hargraves' theory of crustal evolution⁴ is under debate⁵, his model based on estimates of sedimentary rock volumes through time suggests that the actual time of continental emergence was 900–600 Myr ago, which would directly have affected late Precambrian–early Cambrian tidal friction rates. Changes in the Earth's moment of inertia I or the gravitational constant G may have taken place, although it is questionable whether these could be of sufficient magnitude to add significantly to the effects of tidal friction. In any case, our basic conclusion stands: if the nautiloids are valid geochronometers recording the

changing lunar synodic month, then the Moon was very much closer in Ordovician time.

Note added in proof: We do not know how Runcorn obtained his value of 150. After considering all of the possible solutions to the equation, we must reject immediately Runcorn's solution, yielding a Moon further from the Earth, because of its geophysical impossibility. Sufficient data allow for a fairly good understanding of present and near-past recession rates but not for extrapolation of the recession rate of 500 Myr ago. Our nautiloid data provide greater insight into the changing Earth–Moon distance than do any other geochronometers that have been proposed so far.

STEPHEN M. POMPEA

PETER G. K. KAHN

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KAHN AND POMPEA¹ claimed that daily and lunar monthly growth records are preserved in the shells of *Nautilus*. Apart from the geophysical interpretation reached, they fail to present adequate biological analogies and assumptions. As emphasised by Clark², the application of growth lines to palaeoecology must follow a discrete distance behind investigations of growth line formation in modern environments; however, Kahn and Pompea have skipped ahead. An examination of the evidence, which they suggest indicates the timing (lunar monthly septa and daily growth lines), seems appropriate.

(1) Bivalve molluscs show tidally induced skeletal banding, however, these organisms are inhabitants of the intertidal benthos, while *Nautilus pompilius* is pelagic and epi-benthic. The environmental parameters experienced by these two groups are quite different.

(2) The present theory of growth line formation³, although applied to molluscs in general, is based on experimental evidence from bivalves. The external morphology and internal anatomy of cephalopods are distinct from bivalve molluscs. Further studies are needed to implicate 'anaerobiosis' as causing growth lines in cephalopods.

(3) Experimentally reared *Sepia* and *Sepiella* (Coleoidea) show daily lines; these cuttlefish are different, behaviourally and morphologically, from *Nautilus* (Nautiloidea). In view of the many environmental parameters reflected in the growth lines of bivalves this analogy seems weak.

(4) Kahn and Pompea relate the formation of the septa (chamber wall) to a lunar monthly periodicity, while neglect-

Table 1 Relation between length of the synodic month s and the Earth–Moon distance r for constant G and I

s	$\beta = 1/5.1$		$\beta = 1/3.4$	
	$(r/r_0)_1$	$(r/r_0)_2$	$(r/r_0)_1$	$(r/r_0)_2$
30	0.57	0.99	0.53	0.99
27	0.49	1.06	0.46	1.05
24	0.43	1.11	0.40	1.10
21	0.37	1.15	0.35	1.14
18	0.32	1.19	0.30	1.18
15	0.27	1.23	0.26	1.21
12	0.22	1.26	0.21	1.24
9	0.18	1.29	0.17	1.27

ting available published data which relates the septa in *Nautilus* to time: 'From the pressures of gases found within the chamber of an immature animal it is calculated on the basis of reasonable assumptions that a new chamber is added about every fortnight'⁴. If the number of growth lines per septa remains constant throughout ontogeny, as Kahn and Pompea state, why do they not use the fortnightly timing (14 d) to which the 'immature animal' septa correspond?

(5) Finally, they report: 'until now, they (Nautiloids) have not been investigated as possible geochronometers'. However, two specimens of Nautiloids were used in such a way by Pannella⁵ in 1972.

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KAHN AND POMPEA REPLY—As Hughes pointed out, Clark's emphasis¹ that the application of growth lines to palaeoecology should follow a discrete distance behind investigations of growth line formation is certainly valid in its own right. However, until extensive observations of *Nautilus* growth *in situ* are made and the growth rhythms present are delineated, our lines of evidence remain reasonable and viable.

The points raised by Hughes require responses in the same order. (1) Bivalve molluscs of the intertidal regime show large-scale fluctuations in growth due to the variable pressures of the niche they inhabit. Moreover, the tidal response is found to depend on latitude, height of tides, and so on². In an attempt to avoid these problems inherent in the use of intertidal 'living fossils' as geochronometers, we looked at the free swimming *Nautilus*.

(2) Although the phenomenon of diurnal migration of *Nautilus* is still under discussion, anaerobiosis³ seems quite plausible in conjunction with daily vertical migratory habits. However, we agree with Hughes that further studies on the cause of growth line formation in cephalopods are desirable.

(3) The internally shelled cephalopods *Sepia* and *Sepiella* are, along with *Spirula*, the closest living relatives to *Nautilus*. *Sepia* and *Sepiella* migrate diurnally, secrete daily growth lines, and secrete regularly spaced chambers^{4,5}. Thus, they may not be as different from *Nautilus* as Hughes suggests.

(4) The estimate by Denton and Gilpin-Brown⁶ is based solely on gas content of the chambers, and not on any direct growth observations. This theoretical estimate for rate of *Nautilus*

septation⁶ has been considered to be on the extreme low side⁷. Furthermore, Martin *et al.*'s⁷ derivation of a minimum average lifespan involves a septation rate of 30 d per chamber.

(5) Although Pannella did observe the bunching of growth lines on ribs of two Silurian specimens⁸, we doubt that his specimens fulfill the criteria for geochronometry⁹. In addition, Pannella's postulate⁸ that what appear to be ribs on an unidentified Devonian cephalopod are expressions of seasonal growth cycles remains unsubstantiated. An alternative view of what Pannella observed might involve the inherent physical properties of the shell-secreting mantle. Should the mantle which forms the growth increment have to expand radially to cover the morphological ornament (rib), the growth increment can be expected to be smaller than in the valley, where the mantle can stretch further towards the aperture and thus secrete a broader growth increment¹⁰. Thus what Pannella observed was probably not related to any geophysical stimulus.

In conclusion, our initial hypothesis¹¹ may be supported by a recent study of live *Nautilus* kept in aquariums⁷. Although the animals kept in captivity experienced abnormally high water temperatures, and although 'the animals were disturbed too frequently for ideal growth,' Martin *et al.*⁷ note what they deem to be the best growth data obtained in their work: weight versus time growth plots reveal a cyclic change in weight due to the addition of a new shell chamber. In particular, for most of the animals' growth periods investigated, 'the inflection in the growth curve may be seen to recur at intervals of about 30 d'⁷. These results further underline the plausibility of viewing chamber formation as specifically circa lunar synodic monthly.

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PETER G. K. KAHN

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THE study of the shells of molluscs often promotes assumptions about the periodicity of growth lines and other repeating skeletal features. When these are used to draw conclusions about the lengths of past geophysical cycles, difficulties arise. The recent paper by Kahn and Pompea¹ is an excellent example. They claim that shell growth features are of known periodicity

in living nautiloids and, by extension to fossils, can be used to interpret the dynamical evolution of the Earth-Moon system over the past 420 Myr. Their conclusion, that the Ordovician had eight days per lunar month and therefore an Earth-Moon distance only 0.4 its present value, rests on three assumptions: (1) Recent *Nautilus* forms one growth line per day; (2) *Nautilus* deposits a septum sealing off a chamber containing 30 growth lines once every lunar month; and (3) these same periodicities controlled shell growth in nautiloids from the Ordovician to the Recent. We know of no empirical evidence to support these assumptions. On the contrary, empirical evidence exists that makes all three assumptions highly questionable.

Shell growth studies have not been reported for living *Nautilus pompilius*, the

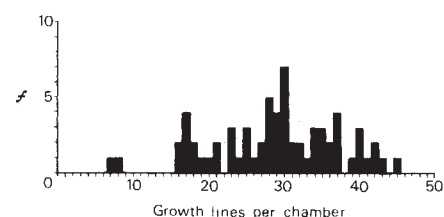


Fig. 1 Number of growth lines per chamber plotted against the chamber frequency for Recent *Nautilus macromphalus*. Data are from Table 2 of Martin *et al.*².

species used by Kahn and Pompea¹, but the closely related *N. macromphalus* has been studied in captivity. Martin *et al.*² observed *N. macromphalus* for several months; on average, one growth line formed every 2 d but with considerable variability. Growth lines were neither daily nor periodic. In addition, counts of the number of growth lines per chamber in 66 chambers of nine individuals showed a wide range, from seven to 45 lines (Fig. 1). Chambers counted ranged in position from second to thirty-first. Early chambers tended to have fewer growth lines than later ones but wide variability existed in the counts. In *N. macromphalus* there is no consistency in the number of growth lines per chamber and, at least in experimental conditions, growth lines do not form daily.

N. macromphalus was studied in captivity; perhaps in natural conditions growth line formation would have been periodic; however, almost all the chambers counted did form in natural conditions before capture, so even if growth lines were periodic, the number of lines per chamber would not be regular and septal formation would seal off chambers with widely varying numbers of growth lines.

The results of Martin *et al.*² do not rule out the possibility that septal formation is periodic. Variation in the number of

growth lines per chamber (Fig. 1) could be caused by several factors. Expansion of the living chamber results from shell growth at the aperture whereas septa form in the most distal part of the living chamber, sealing off shell deposited several months earlier. Therefore changes in growth rate and/or distance between the growth lines can cause variation in the number of growth lines per chamber even with periodic deposition of septa. This is true whether or not growth lines are periodic.

Because the relationship between septa and growth lines is considerably more complex than Kahn and Pompea¹ suggest, both growth line and septal formation may be aperiodic features while the number of lines per chamber still remains fairly constant. This would be true if growth lines are analogous to aperiodic ornamentation in bivalves, which can have regular spacing without consistent relationship to time. Examples are periostracal frills in *Arctica islandica* and surface frills in *Chione cancellata* (I.T., unpublished). If chamber width is also regular, then a repeating number of lines would be found in chambers. Spacing of both growth lines and septa may increase with ontogeny without changing this relationship.

However, *N. macromphalus* does not show a repeating number of lines per chamber² and we doubt that *N. pompilius* does either. Although Kahn and Pompea¹ state that throughout ontogeny 30 lines are present per chamber in *N. pompilius*, the most chambers they seem to have counted in a single specimen is eight. Mature specimens have over 30 chambers. Because only one of the Recent specimens was a juvenile, why were more chambers per individual not counted, and what is the basis for always assuming 30 lines per chamber?

Even if we admit that *N. pompilius* does form daily lines and the animal manages somehow to deposit septa so that 30 of these lines are sealed off into chambers, the case for using fossil species to determine Earth-Moon relationships is still very weak. *N. pompilius* and *N. macromphalus* are closely related species living in similar habitats. If growth in *N. macromphalus* is so different from that in *N. pompilius*, how can we extrapolate from *N. pompilius* to species living hundreds of millions of years ago in very different habitats? Furthermore, there are very few actual data on fossil species—much fewer than a superficial glance at Table 1 might suggest. For most of the periods for which there are any data, only one species was used and very few chambers were counted. To say that 'many of the nautiloids living at any given time secrete the same number of growth lines per chamber'¹ is a gross overstatement of the case.

Another reason for doubting the conclusions of Kahn and Pompea is the subjectivity of growth line counts when

made by eye by researchers with an interest in the outcome. We use their Fig. 2 as an example. There are, they contend, 30 ± 2 lines between the arrows. But we can count a minimum of 24 lines and a maximum of 38 because the lines are of varying prominence.

Kahn and Pompea propose that the rate of recession of the Moon shown by the slope of the line between Recent and the Miocene in their Fig. 6 for the recent past is ~ 25 times the directly measured rate³. One may argue that ocean-continent conditions are poorly known in the Palaeozoic or even the Mesozoic and may have caused increased recession rates. It is quite another thing to account for the gross discrepancy between the directly measured recession rate and the rate proposed for the past 25 Myr.

We thank D. Baird, F. A. Dahlen, A. G. Fischer, and B. Saunders for discussions.

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KAHN AND POMPEA REPLY—As we clearly stated¹, *Nautilus* growth has never been studied *in situ*, thus necessitating our reliance on a study of growth lines. Although Jones and Thompson refer to the investigation by Martin *et al.*² of *Nautilus macromphalus*, they totally ignore the growth data which even Martin *et al.* note as the best obtained in their work. The evidence of Martin *et al.* strongly supports cyclic chamber secretion at ~ 30 -d intervals. Furthermore, Jones and Thompson's proposal that *Nautilus* growth lines and septa represent aperiodic ornamentation is irreconcilable in light of the irregular spacing of growth lines. Finally, different views of what actually constitutes a growth line seem to be involved; indeed, a definition is warranted.

Exponential growth of the *Nautilus* shell entails a constant rate of septa formation³. Martin *et al.*² confirm that growth is exponential and imply that septation is periodic by using a growth rate of 30 d per chamber to derive a minimum lifespan. As for the exact delineation of a septation rate, Martin *et al.*² stress that their results are reported with caution. Moreover, they note the possibility of inherent errors in weighing and counting, that sawing the shell affected ensuing growth, and that 'the animals were disturbed too frequently for ideal growth'. The specimens kept in captivity

experienced abnormally high water temperatures, causing some to cease growing and others to die. All of the calendared date comparisons to determine growth line periodicity were made on shells kept in the aquarium, and all showed roughening (A. W. Martin, personal communication). Should these problems with their obvious effect on growth continuity be neglected, then the data on periodicity of growth lines cannot represent more than estimates, as conceded by Martin *et al.*²

Martin *et al.*'s observations that internal lines (lirae) in the initial five chambers correspond to growth lines on the shell exterior agree with our views¹, and in both studies the consistent number of lirae approximated 30. Chambers six to eight and the last chamber are exceptions to the general rule of increasing distance between septa throughout ontogeny. Therefore, we did not include counts of growth lines per chamber length for those particular chambers. Eliminating the growth line counts of those chambers from the histogram presented by Jones and Thompson would remove the most extreme counts. Despite all of the growth problems related to captivity, it remains interesting that the spread in their histogram concentrates near 30 growth lines per chamber.

Growth lines laid down in the early and middle lifespans of our *Nautilus pompilius* shells seemed to be 'normal', that is, as broad external lines of irregular spacing with a corrugated ridge easily discerned with scanning electron microscopy. Many growth lines formed in the late period of the animals' lifespans were considered 'abnormal' due to compaction and discontinuity of lines as well as numerous breakage and healing marks. As we have clarified¹, growth lines were not counted on fossil and recent shells that possessed breakage scars. It may be argued that an insufficient number of specimens were investigated; however, the unknown time variable involved in shell repair of damaged specimens as well as dissolution of the aragonitic outer shell layer of fossils precluded the counting of growth lines for many specimens. Finally, *Nautilus* coils convolutely, and each whorl encloses the previous one so that no more than eight chamber spaces of the outer shell are countable. These are the reasons why more chambers per individual could not be counted. Our records were obtained by 3–5 impartial observers counting from high resolution photographs or directly from the specimens. We regret that Fig. 2 was not detailed enough to allow Jones and Thompson to accurately 'eyeball' the count.

Using SEM a *Nautilus* growth line appears as a crenulated or corrugated line much brighter or darker (depending on orientation of specimen) than other areas of the shell. The line, an asymmetric ridge between successive growth increments

then represents the structural boundary of two terraced growth increments that were deposited with substantial overlap like shingles on a roof.

As we stated¹, no time direct relationship exists between growth line secretion at the aperture and septum deposition in the distal portion of the body chamber. The relationship between septa and growth line features has never been suggested to be anything but complex. To postulate that septal formation may be aperiodic ornamentation should be done only with great caution, particularly in light of functional morphological^{4,5} and exponential growth deductions³. A constant number of growth lines per chamber might be considered to be the consequence of aperiodic growth line and septal formation if growth lines were regularly spaced, septa were regularly spaced, and growth line breadth expanded during ontogeny in relation to increases in chamber lengths. Indeed, regularly spaced morphological structures (ribs, not growth lines) observed on some nautiloids and ammonoids could well be aperiodic ornamentation. The regularity of such ornaments suggests rhythmic secretion and seems to exclude the influence of fluctuating environmental factors⁵. Unlike growth lines, such ornamentation is generally reflected throughout several shell layers and, of course, lacks a hyponomic sinus. Several examples from the specimens studied may help in understanding a pyritised ammonite specimen of *Lytoceras heterophyllum* (Princeton collection) reveals a constant number of such ornaments per chamber with ontogeny. On the ornamentation and in part oblique to it, irregularly spaced, minute increments of the outermost shell layer are the growth lines. Of the specimens discussed in our article¹, *Reticyclocceras imoense* provides an excellent example of the implausibility of viewing growth lines and septa as aperiodic features. The highly irregularly spaced growth lines can be seen overlying regularly spaced morphological structures. However, the number of growth lines per chamber, for all observable chambers, or this specimen is constant at 15 ± 1 . Note also that recent *Nautilus* specimens can possess quite irregularly spaced growth lines, possibly reflecting short-term environmental pressures. In conclusion, the most devastating argument against aperiodic ornamentation comes from the recent and fossil evidence itself.

We thank R. Culver, W. Furnish, A. Martin, E. Pompea and P. Ward for helpful comments.

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Protein polymorphism and the rate of loss of duplicate gene expression

A CORRELATION between the rate of loss of expression of a duplicate gene following tetraploidy, and its average heterozygosity, is not necessarily to be expected from the neutral mutation theory as suggested by Allendorf¹. This neutralist interpretation follows from the tacit assumption that the mutation rate to neutral alleles is correlated with the mutation rate to loss-of-function alleles, and this assumption is not necessarily

such as frameshift, chain terminator and regulatory mutations and large scale deletions). It is, therefore, those enzymes with the lowest μ_{loss} , that is, with the loosest stereochemical constraints, that will be expected to retain duplicate activity the longest after tetraploidy, which, on the neutral theory, will not necessarily be those with the lowest levels of heterozygosity in the diploid state.

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ALLENDORF REPLIES—Baker clarifies the types of structural mutations that can affect the loss of duplicate gene expression. I do not believe, however, that his point affects the basic conclusions of my letter¹ for several reasons.

Enzymes do not have the same number of amino acids. Assume two enzymes that have different numbers of amino acids. If all else is equal, the larger of the two enzymes will have both larger μ_{neutral} and μ_{loss} . The larger enzyme will thus tend to have the higher heterozygosity in the diploid state and the more rapid rate of loss of duplicate gene expression.

Enzymes with more amino acids do tend to be more polymorphic in natural populations². These expected relationships could be further tested by comparing null mutation rates for different size enzymes for which there exists suitable assay conditions (for example, xanthine dehydrogenase and alcohol dehydrogenase in *Drosophila melanogaster*³). In addition, the actual rates of loss of duplicate gene expression and molecular weights of different enzymes could be directly compared, if more data were available.

The major point of my original letter does not depend on the expected rate of loss under the neutral model. Rather, the important point is that the observed relative rates for loss of gene expression are incompatible with heterozygous advantage being a general mechanism for maintaining the widespread enzyme polymorphism detected in natural populations.

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Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

correct. The neutral point mutation rate (μ_{neutral}) and the loss-of-function point mutation rate (μ_{loss}) of a polypeptide, along with the partial-loss-or-gain-in-function point mutation rate (μ_{partial}), will be components of the total point mutation rate (μ_{total}), and this will normally be approximately proportional to the number of amino acids in the polypeptide concerned; but the way μ_{total} is divided into these components will depend on the overall strictness of the stereochemical constraints on the amino acid sites, and this varies from one polypeptide to another. Thus, in the special case of polypeptides with the same number of amino acids, if μ_{partial} is small then μ_{loss} and μ_{neutral} will not be correlated but inversely related.

On the neutral theory, the average heterozygosity at a locus in the diploid state is related to μ_{neutral} , whereas the average rate of fixation of non-functional alleles at a duplicate locus following tetraploidy will depend on μ_{loss} (although there will also be a contribution from other kinds of loss-of-function mutations

reviews

Galileo's scientific activity

R. H. Naylor

Galileo at Work: His Scientific Biography. By Stillman Drake. Pp. 560. (University of Chicago Press: Chicago and London, 1979.) \$31.25; £17.50.

In this book Drake sets out to provide a chronological discussion of Galileo's scientific activity from his earliest years to his death. This interesting idea provides a number of insights into Galileo's work not previously accessible to an English audience, and in many instances Drake sheds light on areas of Galileo's work little discussed in earlier biographical studies. A great merit of the book lies in the discussion of the surviving evidence of Galileo's correspondence based on the meticulously prepared National Edition of his work edited by Antonio Favaro at the turn of the century. Drake has provided numerous translations of letters, many of which could not have been seen previously in English. In this, and the translations of other writings, Drake fleshes out aspects of Galileo's activity—such as his work on the hydraulics of flood control—which were never treated by Galileo in his published work. As a result, the book offers new insight into Galileo's relationship with his contemporaries and his patrons, Guidobaldo del Monte and Federico Cesi.

Guidobaldo del Monte recognised Galileo's abilities at an early date, and his influence was an important factor in Galileo gaining his first appointment at Pisa in 1589 and his chair at Padua in 1592. It is also very clear from the correspondence, books and manuscripts of Galileo and Guidobaldo that young Galileo was undoubtedly influenced by his patron, although the full extent of this influence does not emerge in Drake's book. One great problem, left unresolved here because of the inadequacy of the historical record, lies in the uncertainty as to when they met. Drake surmises that they may have met in 1593–94 and again in 1602.

Guidobaldo was a leading mathematician of his time and particularly interested in mechanics. The issues involved here relate to Galileo's adoption of a mode of research in which mathematics and experiment were combined. The earliest evidence of successful empirical theory of motion appears in a letter Galileo wrote to Guidobaldo in



1602. It was this letter that announced the discovery of the isochronism of the pendulum and a law relating motions on planes of differing inclination. This last incident is fully discussed by Drake but other vitally important related aspects of Galileo's and Guidobaldo's work at this period are relegated to footnote references later in the book. It is quite clear that Galileo's early work on the projectile trajectory was initially very closely related to the work of Guidobaldo who on the basis of experimentation, concluded that the trajectory has a form rather similar to a parabola or hyperbola.

Drake's treatment of Galileo's manuscript notes relating to the productive late Paduan phase of his work on motion is incompletely documented and at times highly controversial. (These notes are contained in volume 72 of the Galilean MS.) Thus, the hypothesis that Galileo made a straightforward empirical discovery of the $s \propto t^2$ law of fall in 1604 is advanced on the basis of a set of measurements found on one side of a single undated manuscript, although this implausible claim has already been shown to conflict with a variety of other evidence.

Having discovered the $s \propto t^2$ law, Drake argues that Galileo then considered ways of relating it to the change of speed. He claims that Galileo did not regard speed as changing con-

tinuously in fall. However, Galileo's essay *De Motu*, composed in about 1590, quite clearly and forcefully demonstrates that in such motions speed must change continuously; this would suggest that Drake cannot be correct. Drake suggests that Galileo saw two alternative possibilities of explaining the $s \propto t^2$ law in 1604: either instantaneous velocity increased directly with distance ($v \propto s$) or as the square root of distance ($v \propto \sqrt{s}$). He considers that as Galileo could not measure instantaneous velocity in 1604 he could not easily decide between these alternatives. How then does Drake account for Galileo's choice of $v \propto s$ rather than $v \propto \sqrt{s}$ in 1604? He points out that Galileo—who could not see *a priori* how instantaneous and average velocity were related—could have considered how average velocity changed in successive time intervals according to his $s \propto t^2$ law. This gave successive values of average velocity as the series 1,3,5,7,9 . . . which Drake regards as having indicated to Galileo that instantaneous velocity increased with distance. However, this series reveals a linear increase of average velocity with time, which is something quite different. If Galileo was prepared to regard the increase in average velocity as reflecting the change of instantaneous velocity, he would clearly have thought that velocity increased with time in 1604. Moreover, in developing his first proof of $s \propto t^2$ from the assumption $v \propto s$ (on folio 85), Galileo demonstrates firstly that average velocity is proportional to the square of instantaneous velocity, and then asserts that in natural acceleration equal increases in speed are made in ever smaller increases in time. Such inconsistencies within Drake's interpretation and between his explanation and the evidence, seriously affects the credibility of his treatment of this manuscript material.

The puzzle presented by Drake did not exist in Galileo's mind: as his manuscripts and letters show, he believed that as the percussive effect of a falling body increased with the distance fallen this showed instantaneous velocity increased with distance. It is only Drake's whiggishness that obscures this. We know that Galileo could not measure instantaneous velocity in 1604—he obviously did not—how could he?

It was only much later and after great travail that Galileo began to recognise that velocity must increase with the square root of distance travelled. At the time that this occurred, in 1608, he carried out a number of interesting experiments on the projectile trajectory and the manuscripts suggest that his adoption of the new idea ($v \propto \sqrt{s}$) was associated with this work and with his discovery of the parabolic trajectory. As with the law of fall, Drake insists that Galileo's discovery of the parabolic trajectory was an unanticipated result of an experimental investigation. In view of Galileo's earlier work with Guidobaldo and the identification of the parabola as a possible form of the trajectory, this is hard to believe. The document related to this discovery, folio 116', does undoubtedly reveal evidence of an experiment with projectiles and has been successfully reconstructed to provide data very similar to that on the manuscript. (This is not, however, the case with Drake's claims relating to folio 107' and the discovery of the $s \propto t^2$ law). The experiment on folio 116' involves the horizontal projection of a sphere and is clearly based on the assumption that horizontal inertia is preserved and that horizontal and vertical motions are independent. That Galileo would have perceived these assumptions to imply a parabolic trajectory long before he had constructed and completed this somewhat complex experiment, is inexplicably denied by Drake. He is also unwilling to admit that the links between theory and experiment must have been closer and more extensive than such a simple explanation allows. As a consequence he is unable to provide a convincing interpretation of folio 116'.

In Drake's view Galileo was not thinking of the form of the trajectory at all but testing the principle of the preservation of horizontal inertia. But in that case Galileo would have required prior confirmation or mathematical analysis of the independence of horizontal and vertical motion—and this would have revealed the idea of the parabolic form of the trajectory. This consequence of Drake's interpretation leaves him in an untenable position. Although he denies prior experimental or theoretical work, at the same time he recognises this makes it impossible to decide which particular assumption is being tested on folio 116', it also leaves unresolved how Galileo could have established assumptions not tested on folio 116'.

Here, as elsewhere, Drake's interpretations of the manuscripts show signs of modification in the light of criticism but regrettably no references to these alternative interpretations are provided. Unhappily, in attempting to

adapt his position in the light of criticism, Drake's interpretations become extremely confused, and this section of the book seems likely to leave most readers quite baffled.

Drake's treatment of Galileo's career following his telescopic discoveries in 1610 makes much easier reading. His work in this period, when he turned away from mechanics to astronomy, has of course been quite fully discussed in some of Drake's previous articles and books, so there is little that is essentially new in the treatment of the major issues. What the book does provide are new discussions of Galileo's relationships with his contemporaries and translations of little known essays.

One of the most interesting and disputed aspects of this period is the relationship between Galileo and Kepler. Throughout his life Kepler gave his wholehearted support to Galileo's efforts in astronomy but Galileo seemed to be almost totally uninterested in Kepler's work. Yet Galileo knew Kepler to be an outstanding and influential astronomer, as it was to him in particular he turned for support regarding his telescopic discoveries. There were immense differences in outlook between these men but this hardly seems to explain Galileo's blindness to Kepler's concrete achievements. Why was it that Galileo so completely ignored Kepler's discovery of the elliptical form of the planetary orbits? Federico Cesi brought this important discovery to Galileo's notice in 1612. What is more, Galileo knew perfectly well that single concentric orbits were quite incapable of explaining the phenomena and yet it was just this crude idea he later advanced in the *Dialogue on the Two Great World Systems*. Drake believes that Galileo's rejection of elliptical orbits was merely

a result of his lack of interest in small deviations. This view, that only small deviations were concerned, is explained rather simplistically by the argument that the inspection of the largest scale maps of the Solar System will not reveal any great variation of planetary orbits from circularity. But such deviations are quite irrelevant as the deviations that concern astronomers are not on maps but in the sky—and quite unmistakable. It was these very large deviations that Galileo knew of—and ignored in the *Dialogue*. Some better explanation needs to be provided for the curious response on Galileo's part, particularly as it seems that there would have been an advantage for heliocentric theorists to relate the elliptical orbits to the Sun. As has been suggested in the past, some intellectual rivalry on Galileo's part cannot be ruled out—and this does not reduce simply to differences in view with Kepler. Kepler's work was based on the accurate observations of Tycho Brahe whose reputation in this field was unsurpassed but whose ideas we know Galileo thoroughly detested.

As an interpretation of Galileo's work this book is undoubtedly seriously limited but the book nevertheless provides some very valuable discussions of many aspects of Galileo's work. A general deficiency of the book is that the bibliographical and literature citations are inadequate for a work of its kind; as a consequence the book does not reflect the state of research on several topics. Curiously Drake refers in a general way to some of this literature in his preface but subsequently neglects to cite it in relation to the text. □

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Field studies in primatology

Primate Ecology: Problem Orientated Field Studies. By R. W. Sussman. Pp. 586. (Wiley: New York and Chichester, UK, 1978.) £9.50.

THIS indexed collection of 28 papers (all except one previously published) will be of particular value to those who do not have easy access to a good range of journals and specialised primate publications. The first part includes 16 papers (representing 40 species) divided into four sections: Prosimians, New World Monkeys, Old World Monkeys, and Apes. Each section is preceded by a brief but useful introduction to the animals and to their distribution, general biology and behaviour. As the subtitle notes the papers are orientated

towards the study of specific ecological problems, in contrast to the earlier natural history type of study. Most are quite recent—three-quarters of them were originally published after 1971. Over half of them discuss the behaviour and ecology of sympatric species of Lorises (P. Charles-Dominique); *Lemur* (R. W. Sussman); *Callicebus* (W. G. Kinzey and A. H. Gentry); *Cebus* and *Saimiri* (R. W. Thorington); *Ateles*, *Alouatta*, *Cebus* and *Saimiri* (L. L. Klein and D. J. Klein); *Colobus* (T. T. Struhsaker and J. F. Oates); *Theropithecus*, *Papio* and *Cercopithecus* (R. I. M. Dunbar and E. P. Dunbar); *Hylobates* and *Symphalangus* (D. J. Chivers). Papers by A. H. Booth and by J. S. Gartlan and T. T. Struhsaker discuss the particularly rich West African primate faunas. The remaining papers are devoted to *Pongo* (D. A. Horr), comparisons of

Pan and *Gorilla* (V. Reynolds), intra-specific variation in *Propithecus verreauxi* (A. Richard), and *Papio* (T. E. Rowell; R. S. O. Harding; S. A. Altmann).

The second part of the collection documents how the primatologists' attitude to the study of behavioural ecological relationships has changed since the 1960s. In 1963, as the results of the first modern field studies of monkeys and apes were being interpreted, Irvén DeVore (in a paper reprinted in this volume) wrote that the observations suggested "a close correlation between ecological adaptation and the morphology and behaviour of the species". As more information became available, the desire to examine the nature of such correlations rapidly became a dominant theme in primatology and numerous projects were initiated on previously unstudied species. Unfortunately, instead of taking the opportunity to assess the progress of the field and to summarise its changing moods, the editor provides only a brief introductory section. A detailed summary of the field's successes and failures would prove invaluable for those approaching this literature for the first time. Nevertheless the twelve selected theoretical papers, presented in chronological order, provide an excellent review.

Probably the most influential and stimulating of the early papers was published in 1966 by J. H. Crook and J. S. Gartlan on the "Evolution of Primate Societies". Considerably influenced by Crook's earlier ornithological work, this paper proposed several grades of primate social organisation representing levels of adaptation to contrasting environments. As new studies became available it became clear that the grade system required revision to include the forest cercopithecines and the key role of phylogeny. Two papers discussing this are included here (T. T. Struhsaker; F. P. G. Aldrich-Blake).

It was perhaps predictable that the next step would be to construct more formal models showing the relationship between the relevant interacting variables and to re-emphasise the role of sexual selection (W. W. Denham; J. D. Goss-Custard, R. I. M. Dunbar and F. P. G. Aldrich-Blake). This was also done by J. H. Crook in a paper not included in this volume. In each case formalising the issues underlined the inadequate nature of the available ecological evidence (for example, of predation and food distribution). The next attempt at a grand synthesis came in 1972 when J. F. Eisenberg, N. A. Muckenhirn and R. Rudran presented a major review and extension of the grade system. This attracted less attention than one might have predicted, probably because there was

already a movement away from looking at the problem on such a large scale.

Many field studies had started to contrast the behaviour and ecology of sympatric species and it soon became apparent that gross classifications of ecology and social organisation were unsatisfactory. Attention switched to understanding the adaptation of each species to its own niche and to the detailed measurements necessary to document the relationship between diet, ranging patterns, group size, sex ratio and so on (T. H. Clutton-Brock; C. M. Hladik). Two components of the story remain. The first (represented by papers by S. A. Altmann and by J. H. Crook, J. E. Ellis and J. D. Goss-Custard) involves further efforts to make predictions about the effects of particular selection pressures and to

build up a systems approach. To be effective this will have to be integrated with an approach that is not represented in this volume. This is illustrated by the recent attempts of L. B. Jorde, K. Milton, T. H. Clutton-Brock and their colleagues to establish generalisations by investigating the relationship between variables, using quantitative measurements derived from the growing number of high-quality field studies.

Most of these papers are very stimulating. Read critically, however, the enormous gap between theory and evidence is often dominating. The gap is slowly closing, but there is still a long, long way to go.

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Molecular basis of protein function

Molecular Interactions and Activity in Proteins. (Ciba Foundation Symposium No. 60 (new series).) (Excerpta Medica: Amsterdam, Oxford and New York, 1979.) \$28.50; Dfl64.

For the past 30 years, the primary aim of the Ciba Symposia has been to promote international cooperation in medical and chemical research. In this particular proceedings volume, there is more emphasis on chemical rather than medical aspects. The general philosophy seems to have been based on the idea that some deeper insight into the molecular basis of protein structure and function will provide us with answers to medical problems in the future.

In contrast to earlier Symposia devoted to more or less the same subject (*Disc. Faraday Soc.*, 1958 and 1965; Ciba Symposium 1965; Moshach Colloquium, 1972; and so on), no attempt is made to develop a general concept of intermolecular forces, as was intended by Bernal, Caspar, Waugh, or Kauzmann in their classical papers. Detailed information with respect to specific systems seems to have become so overwhelming that a new synthesis is not feasible at the present stage. One reason for this becomes clear when one looks at the two different approaches which contributed most to our understanding of molecular interactions and activity in proteins—namely, X-ray crystallography and nuclear magnetic resonance (NMR). The differing emphasis of the two methods has created disagreement in the interpretation of quite a number of results. What now seems to be most exciting, as seen in many contributions

to the symposium, is the fact that the different groups of workers have started to appreciate the results of their opponents; and what seemed to have been contradictory is now found to be the result of differing methodologies. Crystallographers, observing definite structures, emphasise the functional role of their static picture, whereas NMR spectroscopists, from the observed evidence for flexibility, emphasise the importance of movement. D. M. Blow, C. C. F. Blake *et al.*, G. C. K. Roberts *et al.*, and M. Halsey *et al.* give beautiful examples to illustrate this complementarity using a wide variety of systems (serine-proteases, tRNA synthetase, dihydrofolate reductase, haemoglobin, lysozyme).

In the case of multifunctional systems, several conformational states of the enzyme are suggested; different ordering of different parts of the polypeptide chains may be involved in the expression of different types of catalytic activity. As a general principle, it seems that a highly immobilised structure exists at the active site of an enzyme, providing reactivity and specificity, whereas activation or control are correlated with transitions that involve changes in the degree of order of the enzyme.

In the given context, the functional role of the dynamics of local conformations is discussed in a most lucid way by B. L. Vallee and J. F. Riordan, applying a wide variety of spectroscopic techniques, including stopped flow and relaxation kinetics. The fact that the three-dimensional structure in the crystal and in solution may not always be identical, as evidenced by marked differences between the kinetic properties of, for example, hexokinase, phosphorylase and carboxypeptidase, may be deduced from these studies.

this again puts a question mark on one of the central dogmas of enzymology.

One of the highlights of the volume is F. M. Richards' paper on "Solvents, Interfaces and Protein Structure". Here, packing densities and packing defects are related to the minimisation of surface area on the one hand, and to possible structural fluctuations on the other. Consequently, the interfacial surface tension is used as a parameter, and surface curvatures in the crevices of enzymes are discussed in terms of increased water fugacity which may contribute to the energy of ligand binding.

It is impossible to give an adequate summary of all the 14 articles and their respective discussions. My selection necessarily reflects personal preferences and this applies to the omission of important facets. In this connection, conformational calculations and studies correlating protein folding with associa-

tion might have deserved consideration.

Although no fundamentally new questions are put forward, and no final solutions to unsolved old problems are given, a great number of exciting details are to be found in the articles and their attendant discussions. The book is indeed a goldmine, providing the reader with a distillation of material from many scattered papers. As J. D. Bernal put it: "You may read one in a 100 of papers and you will find out the state of the field by getting a good random sample; the advantage of a good meeting over that method is that the sample is equally small, but it is far from being random". The present one provides the essence of our knowledge in the field of protein research.

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Inorganic and organic luminescent systems

Luminescence Spectroscopy. Edited by Michael D. Lumb. Pp. 375. (Academic: London, New York and San Francisco, 1978.) £24; \$49.75.

THE principal concern of this text is with emission spectra of inorganic and organic luminescent systems. It is a multi-author volume and shows the usual problems of coordination between the contributions, although overlap is not too marked. Two initial chapters introduce the reader to the main features and types of luminescent systems of inorganic and organic composition respectively. Chapter 1 distinguishes the main features and origins of emission spectra and describes the major categories of luminescent solid encountered. A review of the models for analysis of the relatively localised centre systems, for example, first and higher transition element ions dispersed in a matrix, is followed by consideration of the radiative transitions in semiconductors. There is in the former case a greater emphasis on the first transition ions, such as Cr^{3+} and Mn^{2+} , than on the divalent and trivalent rare earth ions, although research on the latter, with their fascinating phenomena of multiphoton and multiphonon transitions and their use for lasers, has been very extensive in recent years. There are one or two inaccurate statements to be found, for example, multi-

phonon processes are already relatively small compared with radiative transitions when 5 or 6 phonon sequences rather than when 25 or so phonon steps are involved. Again (p67), the example of low temperature emission of Co^{2+} in KMgF_3 as a limiting case for 3- μm emission compared with non-radiative loss can be countered by the case of Co^{2+} in ZnS , which gives efficient emission at room temperature.

Chapter 2 gives a brief coverage of the important features of luminescence in organic molecules, including topics such as state crossing, concentration quenching and electronic-vibronic interaction. It points out that the configurational coordinate model needs the additional assumption of tunnelling processes between states below the energies for interstate 'crossovers', a point neglected in chapter 1. Readers should note that the inconsistent use of definitions for fluorescence and phosphorescence between organic and inorganic fields persists, a matter touched on by the authors of chapter 3. The latter chapter contains a very useful survey of experimental methods and instruments for luminescence spectroscopy in use for many years but it also includes modern developments. The great development in luminescence has been the laser and the availability of high power sources of CW or pulsed coherent radiation. However, there has also been the development of single photon counting and weak signal recovery systems, together with time resolved and derivative spectroscopy all of which have given a new dimension in instrumentation to luminescence research. One would have liked to have seen more space given to the varieties

of laser systems for excitation and analysis of luminescence and related effects, which have benefited inorganic and organic research alike, and also the photobiological field, for example, transient absorption spectroscopy which does get some mention.

It is in chapters 5 and 6 that we find the current emphasis in spectroscopy of luminescent systems, namely the combination with magnetic fields to take full advantage of Zeemann effects. Chapter 4 is concerned with organic aromatic crystals and is mainly devoted to the theoretical treatment of the triplet-triplet interactions and the setting up of the requisite spin-Hamiltonian. The reader will require a jump in thought here as the introductory chapters will not have given the more advanced basis needed. However, the treatment is systematic and clear for those who work through it. At the end there is only a small fraction of the text for applications; but these are important cases, such as triplet-triplet fusion, formation of 'exciton cages' and the part played by such processes in electroluminescent organic solids. There is a large field of interest nowadays in bi-exciton and droplet formation by condensation at very low temperatures but perhaps the reader will have to look at current papers both for the organic and inorganic examples. In the final chapter the author covers some aspects of magneto-optical spectroscopy among the inorganic crystalline systems. One sees the force of including the effect of magnetic fields in this chapter in distinguishing the various types of bound exciton and transitions in the donor-acceptor complex. Notable features of the methods are the detection and analysis of electron spin resonance by way of its effect on the emission spectra and the use of the magnetic field to sort out by polarisation the transitions in spectra otherwise not sufficiently resolved to show Zeemann components. Again, as in the case of laser development, the development of the superconducting magnet as a manageable laboratory installation has given a significant extension to the measurements.

The book has a good subject index and each chapter has a bibliography and reference list. It will fill a need among graduate students entering the respective fields of luminescence research or that of higher band gap semiconductors. However, with the price tag, which is not unusual now for such bound volumes, they will have to seek it in a library.

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